

# The end of the nuclear threat?

The Soviet Union (if that name is still meaningful) has replied positively to President George Bush's promise of substantial arms reduction, but that does not mean that the threat of nuclear war has disappeared.

PRESIDENT Mikhail Gorbachev has predictably replied to the US offer of substantial reductions of strategic arms with a promise of matching reductions in the East. Last weekend, he offered to get rid of much the same range of weapons as those to be phased out in the West, throwing in for good measure a year-long moratorium on nuclear tests and a further substantial reduction of the conventional forces at his and his colleagues' disposal. For the time being, these are promises only. The urgent need is that they should now be embodied in a formal understanding. Then there will have to be a formal treaty, which of necessity will supersede that on strategic arms agreed earlier this year in Moscow. While misgivings will persist in the US Senate (which must ratify all formal treaties to which the United States puts its name) about such matters as the decision not to deploy MX missiles on mobile launchers, the new package makes an easier pill to swallow than that earlier offer.

That is the good news. But neither of the two participants in this unprecedented public auction of arms reductions is at this stage planning to dispense with its capacity to deter the other from a nuclear attack. Each will retain a substantial force of strategic nuclear missiles, many of them on submarines at sea or in aircraft ready to take off. The arrangements now proposed will drastically reduce the risk of nuclear war by accident or miscalculation, as well as the danger that conventional conflicts will escalate into a devastating nuclear exchange, but the threat of overwhelming retaliation for a strategic nuclear strike will remain. That, the US Senate will no doubt decide, is also good news.

## Uncertainty

The snag is the persisting uncertainty about the legal authority of the Soviet Union to make international undertakings that are binding on its member states. The meeting at the end of last week of the republic governments interested in continued mutual collaboration appears to have demonstrated that only half of them are likely to become partners in a scheme for continued economic collaboration. Not only the three Baltic states, but also the Ukraine, seem more inclined towards opting out, while republics such as Georgia are so racked by domestic turmoil that they cannot be considered credible members of a durable union. Yet without a stable union, and arrangements whereby taxes can be gathered to pay for

military defence, there can be no central command and control of nuclear weapons, and thus no substantial signatory for a durable treaty. That is why it is now in everybody's common interest that attempts to forge a new union from the rump of Stalin's empire should succeed. That calculation is more likely to unlock Western economic assistance than Moscow's entreaties in recent weeks.

## Problems

Meanwhile, Western Europe is faced with two more immediate problems. The disappearance of US short-range nuclear weapons will knock a hole in the strategy for the defence of Western Europe, which has relied for the past 30 years on the putative use of nuclear weapons to deter incursions from the East. For the time being, at least, the threat has gone, which makes the nuclear weapons pointless. But that does not abolish the need for a strategy of some kind. The issue is further complicated by the decision of the European Communities (EC) that the Treaty of Rome should be amended to allow for greater collaboration on political matters, in principle including arrangements for mutual defence. (There are only six weeks left before the meeting planned at Maastricht at which an amended treaty must be signed.)

One difficulty is the eagerness of some EC members (notably the Netherlands, which holds the chair of EC committees until the end of 1991) to make foreign policy (and so defence) a matter for community decision. Another is the advocacy by others of military coordination through the Western European Union and thus with the North Atlantic Treaty Organization (NATO). Where the European Rapid Reaction Force advocated last week by Britain and Italy would fit in is another source of confusion. The Communities would be well advised not to rush decisions on these issues simply to meet the artificial Maastricht timetable.

And what is the future of the two nuclear powers that are members of the Communities? That is the second problem crying out for solution. London and Paris have reacted instinctively to the prospect of further significant reductions of nuclear weapons by declaring that their own nuclear deterrents will be unaffected. That is understandable, but short-sighted. There are good reasons why both nuclear powers should reconsider their positions, if not now, then soon. For one thing, there is money to be saved. For another, it is no longer self-evident who their weapons



are now intended to deter. And there remains the danger that the potential use of airborne nuclear weapons in Central Europe will impede the beneficent process now under way. □

## Baltimore's defence

Although the final decision in the Imanishi-Kari case is some way off, one issue in the case is already clear.

It will have been apparent, in the past several weeks, that the earlier exchange of opinions by the participants in the Imanishi-Kari case appears to have petered out. This is not because those concerned have run out of things to say, but rather reflects this journal's judgment that its readers have seen the essence of the dispute laid out, and are likely to be confused by ever more detailed statements. But there is one issue, raised by Professor Paul Doty (*Nature* **352**, 183; 1991) which requires a decision by the scientific community rather than by an office of the US National Institutes of Health (NIH): what are the responsibilities of the authors of a published research report?

The issue is simply stated. Dr David Baltimore, the most celebrated although not the principal author of a disputed paper (*Cell* **45**, 247; 1986) has from the outset taken the view that it is for the scientific community at large, and for others working in the field concerned, eventually to demonstrate the validity or otherwise of the disputed data and the conclusions drawn from them. It is a point of view, but hardly a defensible one, especially when the authenticity of the data on which the disputed paper's conclusions were supposedly based has been sharply questioned. Doty is right to complain (on page 495) that this among his questions has not been dealt with by Baltimore (see *Nature* **353**, 9; 1991). Specifically, Doty argues that Baltimore should have repeated the challenged experiments in his own laboratory.

The plain truth is that the authors of all published research reports have a personal responsibility for their aftercare. They, after all, are best placed to carry out the meticulous examination of the original data when questions are asked about them and, when necessary, to design and carry out replicate experiments. Often, if the outcome of such *post hoc* investigations is to undermine the original conclusions, the original authors are much more likely than outsiders to arrive at an explanation of what went wrong.

So much has hitherto been generally accepted. Were it otherwise, science itself would be undermined. For the presumption would be falsified that what appears in the literature can be regarded, at least provisionally, as authentic. People seeking to make use of published data would then have to repeat the experiments before carrying on with their own work. Can that make sense? Naturally, it must often be a fiendish nuisance for authors to have to shoulder this responsibility, especially if many months have passed. But that is merely one of the penalties of authorship. □

## Homeopaths' cushion

The European Commission is planning to give homeopathy an easy ride. It should think again.

THE European Commission (the executive arm of the European Communities) has acquired (probably wrongly) a reputation for being too fussy in its regulation of materials on the European market. The British, for example, have recently fought a battle with the commission over the regulation requiring sausages to be made mostly of meat (whereas, in Britain, their chief constituent is carbohydrate). But the commission is plainly not over-fussy. It now appears to be caving in to pressure from the European Parliament to exempt homeopathic medicines from the strict rules that must be satisfied by conventional medicines, known to homeopaths as 'allopathic'.

It is a curious business. On 13 June, the European Parliament approved what is called the Chanterie report (after the Belgian rapporteur of an *ad hoc* parliamentary committee) with only one dissenting vote. Among other things, the document asks that homeopathic medicines should be exempted from the "conventional statistical methods for clinical trials" and that, instead, there should be a simplified method of registering their availability for sale. Part of the case for exemption from the strict requirements of directive 75/319/EEC is that the active ingredients of homeopathic medicines are so diluted that questions of safety do not arise. But the much more telling consideration is that homeopathic medicines are widely used in many European countries, France and Belgium, for example.

The danger is that under the policies of the EC, homeopathic medicines will spread throughout Europe and Britain to the detriment of an innocent public. EC regulations provide that any item that can be sold in one country can be sold in any other member nation. A French homeopath would have only to open a chemist's shop in London to be in business.

Even those who practise homeopathy should be affronted by these developments. If certain substances can be effective medicines even when diluted to a very high degree, may they not also have unintended side-effects? Conversely, if the same materials cannot by definition have side-effects, what rational assurance can there be that they are nevertheless therapeutic? And, in any case, given the complaints of the homeopaths that their medicines are only rarely subjected to serious investigation, is not the commission's directive precisely the opportunity they need to make homeopathic medicines respectable?

The plain truth is that the European Parliament has not enhanced its poor reputation by nodding through this irresponsible amendment of the drug regulations, and that it will be a disgrace if the Commission now goes on to compromise its drug regulations by unfairly giving homeopathic medicines a fair wind. It should think again. □



# US patent application stirs up gene hunters

■ NIH researcher files for 337 genes at one time

■ When should a gene be patentable?

## Washington

UNTIL this summer, no one worried much about patenting human genes. Cystic fibrosis, muscular dystrophy, Huntington's disease, whatever — map it, sequence it, take it to the patent office. Unless someone complained about apportionment of credit, gene patenting was business as usual.

Then, on 20 June, Craig Venter dropped a 400-page application on the US Patent Office. With a single filing, Venter requested patents for 337 new human genes — DNA fragments that his automatic gene sequencers at the National Institutes of Health (NIH) had cranked out in just months, almost unaided. In a few years, he said, he could come back with most of genes in the entire human genome.

Now, three months later, gene hunters are bracing for a patent 'gold rush'. Researchers around the world have suddenly developed an interest in the working of the Patent Office — as well as in Venter's simple technique, which is based on sequencing only the DNA that is actually expressed in an organism, known as complementary DNA, or cDNA. With a laboratory full of robotic gene machines, Venter can sequence 75 kilobases of cDNA a day, including perhaps 100 new genes. He has virtually no idea what the individual genes do, but they must do something. Any given one of them could be

linked to some form of cancer, Alzheimer's disease or some other genetic defect.

"If these things are patentable, there's going to be an enormous cDNA arms race," predicted Max Hensley, senior attorney for the biotechnology company Genentech, at a gene sequencing conference in South Carolina last month. Hensley thinks that might not be such a bad thing. "If we get an entire cDNA library, what's wrong with that?" But many other influential people find plenty not to like about Venter's work, cDNA sequencing and the prospect of patenting genes by the pound.

"I think it's a terrible idea," says Maynard Olson, a Washington University geneticist and member of the Genome Project's advisory panel. "If the law is interpreted to give intellectual property rights for naked DNA sequences, then the law should be changed. It's like trying to patent the periodic table. To put patent value on cream-skimming is sending entirely the wrong signal. Scientists who advocate such approaches are playing with fire." James Watson, director of the National Center for Human Genome Research at NIH, has called cDNA patenting "outrageous".

Some scientists argue that the wholesale patenting of DNA sequences when their function is still unknown is an abuse of the patent system. "Patent law wasn't designed to be a kind of lottery where one

guesses every large number of letters that might be the right combination," Olson says. Others are afraid of the 'chilling effect' that commercialization of gene sequences so early in the process might have on future industrial investment. Still others are concerned that patenting costs will drain money from research, that researchers may withhold data until their patent is granted or that patents will force countries to compete, rather than collaborate, in the race to lock up the entire genome.

But underlying much of the concern is the sense that large-scale cDNA sequencing is just not sporting. The Genome Project was sold to Congress as a 15-year, \$3,000-million effort to map and sequence the entire human DNA molecule. Now Venter says he can get almost all the genes — the only part of the genome most congressmen care about — in a few years, for perhaps \$10 million. Venter is quick to emphasize that cDNA sequencing is no substitute for mapping, and that finding the location and the function of the genes will be essential to the project. But once that is done, convincing Congress that another \$2,500 million still needs to be spent on sequencing the rest of the genome — the 97 to 98 per cent that contains no genes — might be difficult.

"There's an undercurrent of tension between those who advocate a cDNA approach and those who advocate the heavy-weight sequence-to-the-end approach," says one industry observer. "Venter says he can do it cheaply. But the glory of it all gets dissipated to the wind once you've sequenced all the genes. It's a matter of who leads the project. The patent issue is just a lightning rod on the whole controversy."

For the moment, all eyes are on the

## To patent a naked gene

In biotechnology, as in any other field, an invention must satisfy three basic criteria to win a patent. It must be novel, it must be nonobvious, and it must have some utility.

Craig Venter's patent application for 337 new genes (see above) does appear to be novel. He has searched all the available databases for matches and the genes seem never to have been sequenced before.

But obviousness is a trickier issue. Venter invented neither the concept of sequencing cDNA nor the technology that allows him to do it so easily, and application of known technology in a standard way is classic obviousness. Venter's insight, however, was that large-scale cDNA sequencing, combined with the searching ability of electronic DNA databases, could generate human genes at an unprecedented rate, and that must be worth something, NIH lawyers argue.

"Patent law specifically says that you can't consider how the invention was made," says Reid Adler, the NIH technology-transfer expert who handled Venter's patent. "It's irrelevant if a trained chimpanzee can do the work. The law doesn't want some judge deciding if someone sweated enough to deserve a patent."

Perhaps the toughest hurdle for Venter's sequences is utility. His genes are 'naked' — merely expressed DNA whose function is unknown. "We argue that you could use the sequence as a probe or to distinguish brain tissue from other tissue," says Adler, but he admits that this is less than an airtight claim. For one thing, although all of Venter's cDNA samples come from brain tissue, many of the sequences probably code for common 'housekeeping' genes that can be found anywhere in the body. They would be no help in identifying brain tissue.

"This is not the strongest case for utility I've ever seen, but it's not the weakest either," Adler says. "It was worth filing the application, if for no other reason than not to miss the boat."

Other patent lawyers agree. "I would hate to be the NIH administrator that has to explain why the UK or Japan were able to obtain a patent when the US didn't even try," says one.

Nevertheless, the application is considered a long shot. Although patent office biotechnology expert Charles Van Horn would not comment on the specific application, he raised concerns about the general idea of patenting fragments of unknown genes. "Is it specific [to a particular gene]? Without that kind of information, it is not patentable. In the chemical side, this has been tried — without clear utility — and it hasn't been patented," he says.

C.A.



Patent Office, which must decide whether or not to approve Venter's application (see page 485). But assuming for the moment that a patent is granted, there remains the question of whether patents for unmapped genes are a good thing, in any form. Venter argues that a patent ensures that a gene will be available for all researchers and for any company willing to license it.

Simply placing a sequence in the public domain, as most researchers now do when they publish it and submit it to a database, usually means that no company can get a patent on the gene unless it has near-clinical use, such as identifying its expression products or developing a test based on it. Even then, patents based on 'methods of use' tend to be open to challenge. Few companies are likely to risk millions of dollars in development costs on a gene product they may not be able to patent. On the other hand, if Venter's patent turns out to be just the first of a string of ever more specific patents as the gene is progressively characterized, then that is just one more expense and complication, and another question mark for a prospective investor. "What if the gene turns out to be linked to another gene that the French have licensed?" asks one biotechnology lawyer. "I'm not going to invest a million dollars with that kind of uncertainty."

Since June, Venter has found nearly 2,000 more genes, and a second patent application is in the works, something that seems likely to fan the flames of the controversy. Hoping to air some of the issues, the NIH technology transfer office will hold a one-day conference, to which the public and industry are invited, on 14 November. And in the meantime, NIH is considering asking the Patent Office for expedited consideration of the application, which could generate a decision in six months, rather than the usual two years.

Whatever the conclusion, Venter seems to have made his point: cDNA has a place in the genome project. Next month, the Genome Center will consider its first applications for cDNA sequencing. (The Department of Energy and the National Institute of Neurological Disorders and Stroke, where Venter works, have been funding cDNA work for several years.)

"If the patent office allows Venter's application, by far the greatest return on investment would be to patent cDNA," says Thomas Caskey, a Baylor College of Medicine genetics researcher. "It's a huge gamble to make an error on this." The fact that one-fifth of France's new \$20 million genome effort will be aimed at cDNA sequencing, as is much of the UK, European Communities and Japanese programmes, has not gone unnoticed, either. "If the patent office says yes," predicts Caskey, "then it's Katy bar the door."

**Christopher Anderson**

## In space, no one can hear you oink

### Washington

THANKS to Congress, US astronauts will soon not only have fewer of those annoying science projects to worry about, but they may also have their choice of Coke or Pepsi in orbit. This news may come as some surprise to the crews, however. Not only did the National Aeronautics and Space Administration (NASA) not ask for an 'advanced liquid dispensing technology evaluation', but agency officials know almost nothing about it. So why is it in NASA's new 1992 budget? Because the soft drink manufacturers lobbied key members of the Senate Appropriations committee, who quietly inserted the language last month into the funding bill.

In total, members of the House and Senate appropriations committees put more than a dozen unsolicited projects into the space budget to steer NASA money into their own home districts, a process known as 'pork barrel' funding. Last week, two peeved congressmen finally decided to take them on. In speeches on the House floor, Robert Walker (Republican, Pennsylvania) and George Brown (Democrat, California) savaged their colleagues for earmarking \$137 million in the budget in the same year that they eliminated or delayed at least ten space science projects, claiming lack of funds.

The earmarked projects "were never requested by the administration, never authorized, and never discussed on the floor," complained Brown. Yet the appropriations committees found themselves unable to afford real NASA programmes such as the space infrared telescope, the orbiting solar laboratory, the aerospace

plane and the flight telerobotic server. "These are all projects that scientists have spent decades planning and developing. These are all projects that could have been funded with a little more restraint on the part of the conferees," Brown charged.

Some of the chief offenders on the Brown and Walker pork barrel hit list are:

- \$20 million for the Christopher Columbus Center for Marine Research in Baltimore, Maryland, home district of Barbara Mikulski, chair of the Senate appropriations subcommittee for NASA. Although Mikulski failed to get this in the National Oceanic and Atmospheric Administration budget last year, she had better luck this year with NASA. The space agency is now in the marine research field, whether it likes it or not.

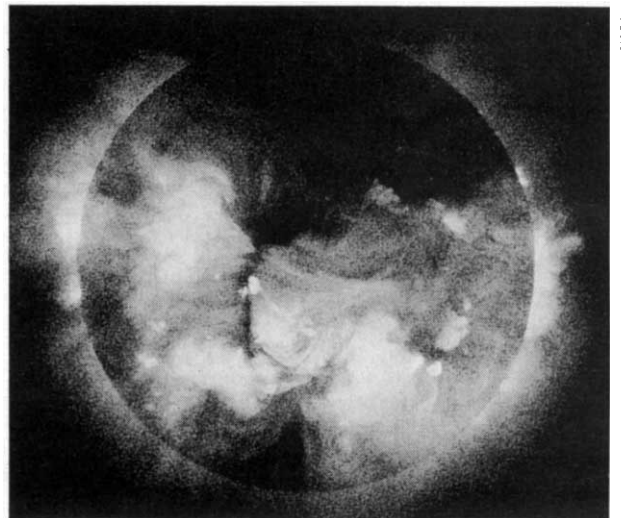
- \$28.4 million for the Consortium for International Earth Science Network in Michigan, home of Bob Traxler, chairman of the House appropriations subcommittee for NASA. Total funding for this project is now more than \$41 million, "all awarded without adequate competitions and virtually no congressional oversight," Brown protested. "NASA itself has little idea where this funding is going."

- \$22.5 million for the National Technology Transfer Center and \$7.5 million to equip a "classroom of the future" at the 1,400-student Wheeling Jesuit College, both in West Virginia, home of Senate Appropriations chairman Robert Byrd, who has promised to bring \$1,000 million in earmarked projects to the state and is well on his way to succeeding. This year's total from the NASA budget alone is \$42 million.

**Christopher Anderson**

## 'Sunlight' sees Sun in a different light

THIS image of the Sun's corona was taken by the Soft X-Ray Telescope aboard the Japanese satellite *yōkō* ('sunlight') after it was launched on 30 August from the Kagoshima space centre in Japan (*Nature* **353**, 102; 12 September 1991). The telescope, a joint experiment between scientists at Japan's National Astronomical Observatory and the US National Aeronautics and Space Administration, reveals details of the X-ray corona. Soft X-rays are produced when the temperature of the gases exceeds about 2 million degrees Fahrenheit. The loop-like structures in the photograph are formed by the Sun's magnetic field shaping hot coronal gases into 'magnetic bottles', and the brightness of the loops indicates the temperature and the density of the gases within them. In visible light, large sunspots would appear under the brightest loops.



NASA

# Patch clamp brings honour

## London

THIS year's Nobel prize for physiology or medicine has gone to Erwin Neher and Bert Sakmann, two German electrophysiologists who in the mid-1970s transformed cell physiology with their invention of 'patch clamp' recording from cell membranes.

Neher and Sakmann made it possible for the first time to record the electrical activity of very small areas of membrane, so that the effects of opening and closing single ion channels could be observed. In their pioneering paper, published in *Nature* (260, 799; 1976), the pair described how a minute area of cell membrane could be isolated electrically by pressing the tip of a glass pipette, less than 5 µm in diameter, against the cell surface. With this pipette fitted with a recording electrode, Neher and Sakmann confirmed that when the membrane potential of a cell alters rapidly — for example, in response to an action potential — ions cross the cell membranes through channels releasing one ion at a time.

The patch-clamp technique revolutionized cell physiology, improving the sensitivity of previous recording techniques by a million-fold. For the first time, physiologists were able to study the minute changes in current (around 1 picoampere) caused by the movement of ions through a single ion channel, essentially studying in real time the activity of the single protein molecule that regulates each channel.

Combining the sensitivity of patch-clamp recording with the techniques of molecular biology, it has been possible to examine how mutations causing single

amino acid changes in membrane proteins affect the behaviour of individual ion channels.

The patch-clamp method has also been used to study the electrical activity of entire cells. By penetrating a cell with a pipette, it is possible not only to make electrical recordings but also to manipulate the internal environment of the cell by adding ions or second messenger molecules. Before the patch-clamp method, whole cell recordings were possible only from extremely large — and by no means typical — cells, such as giant axons in snails and oocytes of sea urchins.

Several recent Nobel prizes have engendered some controversy, notably the claim by the French researcher Dominique Stehelin that he should have shared the 1989 medicine prize awarded to Michael Bishop and Harold Varmus (*Nature* 341, 556; 1989). But Neher and Sakmann's award seems to be refreshingly free from such disputes. "There's absolutely no doubt at all that the credit for this method lies with Neher and Sakmann," says David Colquhoun, from University College London.

The only complaint expressed by some physiologists is that the Germans had to wait so long for their prize. David Clapham, a former post-doctoral researcher in Neher's laboratory now at the Mayo Clinic in Minnesota, recalls from his post-graduate days the immediate impact that the 1976 *Nature* paper had on the field. "We have expected this for almost ten years," adds Clapham's Mayo colleague Julio Fernandez, also a veteran of Neher's laboratory.

Peter Aldhous

## BIOTECHNOLOGY

# French drug industry initiative

## London

THE French pharmaceutical company Rhône-Poulenc is expected to announce a new government/industry biotechnology initiative this month that could amount to between FF 1,000 million and FF 2,000 million (\$170-340 million) in new research funding over the next five years. Known as Bio-Avenir, the project is part of a major shift in the mostly state-controlled French pharmaceutical industry towards more life sciences research — supported by an increase in the price of research-intensive drugs.

Bio-Avenir is to be split between industry and government research institutes, with between 30 and 50 per cent of the funding coming directly from the government. Research priorities are expected to include cellular regulation, the structure and function of macromolecules and membrane transport. Details of the programme

were first reported last month by *Biotechnology Business News*, a Financial Times newsletter. Officials at Rhône-Poulenc and in the French government said that the initiative will be run from Rhône-Poulenc's Paris headquarters, but they declined to comment on other details until the programme is formally announced.

Seeking to bring the domestic pharmaceutical industry more in line with European standards, French cabinet officials are also planning to propose major drug pricing reforms to Parliament in October. If the new plan is approved, French pharmaceutical companies will be able to charge enough for 'innovative drugs' to recover research costs and keep up with their international competitors. State pricing controls now keep French drug prices some 30 per cent below the European Communities average.

Christopher Anderson

# DOE won't wait on WIPP

## Washington

THE US Department of Energy (DOE) announced last week that it will wait no longer for congressional action on its Waste Isolation Pilot Plant (WIPP) in New Mexico, but instead will begin testing at the nuclear waste disposal test project as soon as possible. That may not be too soon, however, as the New Mexico attorney general has said he will seek to block the move in court.

WIPP is the energy department's main research and development facility for the disposal of nuclear wastes from weapons manufacture. It is designed to store approximately 6.5 million cubic feet of waste contaminated by plutonium and other transuranic elements in a salt repository some 2,150 feet underground.

On 3 October, Admiral James Watkins, Secretary of Energy, announced that WIPP is ready to begin testing, and all that remains to be done is to withdraw the land containing the site from the public domain and put it under the control of DOE. The department has been negotiating with Congress on a bill that would provide for the land withdrawal and in return provide some \$600 million to New Mexico in federal benefits.

Those negotiations have been stalled, however, on the question of how much waste WIPP will be allowed to accept in its initial 'performance assessment testing'. On the recommendation of the Environmental Protection Agency (EPA), the energy department insists that it should be allowed to bring as much as 8,500 barrels of waste — 1 per cent of capacity — into the facility. New Mexico senator Jeff Bingaman is demanding that the limit should be 4,250 barrels and that the DOE should be required to obtain approval from Congress to exceed that amount.

In the face of this impasse, Watkins decided to bypass Congress altogether. The WIPP site has been under the control of the Department of the Interior since 1983, and under existing legislation it can be transferred to the energy department without the approval of Congress if the DOE certifies it has met all environmental requirements for WIPP. Watkins sent that certification on 3 October. The energy department would prefer Congress to carry out the land withdrawal, Watkins said, but "not at the cost of running WIPP in an unsound manner".

Although the governor of New Mexico is in favour of proceeding with WIPP, the attorney general — the state's senior law enforcement officer — has said he will bring suit in court to oppose administrative land withdrawal. If he does, it is likely to be an expensive suit: DOE has promised to pay \$63 million to New Mexico once WIPP is under way, but it has said it will withhold the money if the state takes any legal action.

Robert Pool



# Fujitsu and TI fight it out

## Tokyo

MEL Sharp, senior vice president and general patent counsel for the US semiconductor manufacturer Texas Instruments, could barely conceal his frustration at a press conference in Tokyo last week when he described the battle he has fought with Japan for the past 27 years, trying to establish the US company's claim to a fundamental patent for integrated circuits.

The Texas Instruments press conference was held after the first hearings in a legal battle in the Japanese courts between the US company and Fujitsu of Japan over the Kilby patent. Texas Instruments claims that the patent — which describes the first primitive integrated circuit invented by Jack Kilby in the late 1950s — applies to all modern computer chips, and the company is demanding enormous licence fees from Fujitsu and other Japanese semiconductor manufacturers. But Fujitsu says the patent, which was finally granted in Japan in 1989, applies only to old technology no longer in use, and the Japanese company filed a suit in the Tokyo District Court on 19 July seeking a decision that the patent does not apply to Fujitsu chips. On the same day, Texas Instruments filed a *kari shobun* (injunctive relief) action to stop use, production and sale of all Fujitsu integrated circuits.

The patent dispute is crucial for the financial future of the two giant companies. It also draws attention to some of the fundamental differences between the US and Japanese patent systems and illustrates the growing importance of intellectual property rights in the struggle for technological supremacy between the United States and Japan.

Sharp says that when he first visited the Japanese patent office in 1964, he was dismayed to discover that the Kilby patent, filed in Japan in 1960 on the basis of the 1959 US application, was dormant. Nothing had happened for four years. After two days of intensive discussions with Japanese patent authorities, he decided that the only way to proceed was to break the patent up into several divisions because of a very basic difference between US and Japanese patent law.

At the time, under Japanese law, a patent could make only one claim, and, as a result, Japanese patents were (and still tend to be) very narrow in scope. In contrast, the initial Kilby patent awarded in the United States in 1964 had 25 claims. The Kilby invention ultimately led to five US patents with a total of 60 claims.

The first division of the Japanese patent application, numbered 320,249, was published in 1965, and, after opposition by Japanese companies, was eventually granted in 1977; it expired three years later, or 15 years after publication. The

only other division to survive — number 320,275, the one now under contention — was “rejected, and rejected, and rejected”, Sharp says, until it was finally accepted for publication by the Japanese patent office in 1986. The patent was granted in 1989 and will expire in 2001.

The patent focuses on the two-dimensional layout of circuit elements and their interconnections in integrated circuits. Because only one claim per patent was allowed, Sharp says Texas Instruments had to be extremely careful in the selection of words in the patent description, because there was “just one chance” to be right. And the legal dispute between Fujitsu and Texas Instruments in fact centres on interpretation of only one or two words.

For example, where the patent says conductors are “laid down” on an insulator, Fujitsu argues that this means detachable wires on an insulator, as in the original Kilby circuit. But Sharp says it just means “conductor on insulator” and applies to the thin-film coatings of conductors used in modern chips.

At stake in this argument are tens of millions of dollars, and possibly the future of the two companies. Texas Instruments has recently gone into debt as it invests heavily to gear up for development and production of next-generation chips, in particular 16-megabit memory chips. Revenues from patents are a vital source of funds to do this. The company's quarterly income from patents now stands at about \$40 million, according to press reports, much of it coming from Japanese companies that have (reluctantly) accepted the validity of the Kilby patent.

Similarly, Fujitsu is investing heavily in next-generation memory chips to try to catch up with other Japanese manufacturers. And, unlike some of its Japanese competitors, Fujitsu does not have much leading-edge technology to barter in cross-licensing agreements with Texas Instruments. That is why, industry sources say, the company decided to fight in court.

Although other Japanese companies, such as NEC and Toshiba, have entered into licensing agreements with Texas Instruments, many people in Japanese industry are privately encouraging Fujitsu because they think the US company has become too greedy in trying to squeeze money out of old technology.

Sharp, on the other hand, sees the court battle as a test of the fairness of the Japanese patent system. The patent office has been “very fair” since 1986, he says, and “we prefer to focus attention on the past five years rather than the preceding twenty” in the belief a fair decision will be reached.

David Swinbanks

## SBIR is good business

### Washington

As the US government focuses more and more on the goal of commercializing federally funded research, there appears to be at least one programme that is meeting that objective. According to studies by the General Accounting Office (GAO) and the Small Business Administration (SBA) outlined last week, the Small Business Innovation Research (SBIR) programme has been highly successful in channelling research money to small companies that transform the research into commercial products.

Established in 1982, the programme mandates that any federal agency that spends more than \$100 million on outside research must set aside at least 1.25 per cent of its research funds for small companies working on projects with commercial potential. In fiscal year 1990, \$461 million was spent on SBIR grants, more than 90 per cent of it coming from five agencies: the Department of Defense, the National Aeronautics and Space Administration, the National Institutes of Health, the Department of Energy and the National Science Foundation. Since its inception, the programme has directed \$2,200 million to 5,500 small companies.

The programme is scheduled to expire in 1993 if not renewed by Congress.

The GAO found that up to the end of July, SBIR-funded research had led to \$471 million in sales for the companies receiving grants, and two-thirds of those sales have been to customers other than the federal government. The companies reported that they expect another \$1,940 million in sales by the end of 1993. The small businesses have also received extra, non-SBIR funding to develop the results of research paid for with the grants — \$646 million through July, with several hundred million more dollars expected by the end of 1993.

Furthermore, GAO judged that the quality of research has not suffered. The participating federal agencies reported that, on average, SBIR research was as good as or better than other contract research.

The SBA study, although differing in details from that of the GAO, agreed that the programme has been successful in encouraging commercialization of federal research.

One possible problem with the programme is that it could encourage ‘proposal factories’ — small research companies that file dozens or hundreds of SBIR proposals with little interest in commercializing the results. The GAO found some circumstantial evidence of this: on average, the companies that received five or more SBIR grants produced fewer sales and less extra developmental funding per project than did companies with fewer than five awards.

Robert Pool



# Tide turns against LD50

## London

THE end may be near for the LD50 test, the controversial procedure used widely to assess the toxicity of new chemicals, which necessitates the death of large numbers of experimental animals. The Paris-based Organization for Economic Cooperation and Development (OECD) is expected to adopt new guidelines for toxicity testing next month that would replace the LD50 test with a new procedure that looks for the toxic effects of a fixed, but sub-lethal, dose of a chemical on a small group of animals. The OECD guidelines are the leading international standard for toxicity testing, and any changes to them are important because all the developed nations that make up OECD's membership demand stringent tests of a new chemical's toxicity before it can be marketed.

The purpose of the LD50 test is to identify the dose of a chemical that kills 50 per cent of a group of animals. But because that necessarily implies the killing of a large number of the animals (usually rats), the test has long been opposed by animal welfare groups. The first LD50 tests, in the late 1920s, often involved 100 animals each, but it is now more usual to use some 30 animals per test. Nevertheless, Phil Botham, from ICI's Central Toxicology Laboratory in Cheshire, says toxicologists have also become unhappy with the test, and over the past decade have been searching for a less crude alternative.

The impetus for the present moves within OECD comes largely from the British Toxicology Society. In the early 1980s, the society developed a 'fixed dose' procedure, which requires fewer animals to test a new chemical and measures subtle toxicological effects, rather than a straightforward distinction between life and death.

OECD decided to review its guidelines after the publication last year of a trial involving many of the leading toxicology laboratories in Europe, the United States and Japan, which endorsed the fixed-dose method. OECD settled on a test in which ten rats (five of each sex) are given a single dose of either 5, 50, 500 or 2000 mg per kg of the chemical being tested. Botham, who has been advising OECD on the new guidelines, says that an experienced toxicologist can often predict, given some basic knowledge about the chemical being tested, which dose will show some toxic effects without killing many of the rats involved. If the initial dose chosen is too high or too low, a second test with ten rats is run, at a different dose. In practice, testing a new chemical using the fixed-dose method requires an average of 17 rats, says Botham, and deaths by poisoning should be relatively rare — a substantial improvement on the wasteful LD50.

Nevertheless, OECD's attempt to do

away with the LD50 test ran into a brick wall at a meeting in Paris in May, when officials from the United States and Japan unexpectedly announced that they could not accept the specific fixed-dose proposals being put forward by OECD. The problem, says Richard Hill, from the US Environmental Protection Agency (EPA), was that the doses chosen by OECD fit well with European classifications for whether a chemical is 'very toxic', 'toxic' or 'harmful', but less well with the US or Japanese systems. EPA classifies chemicals similarly, but the divisions between the different categories are set at different doses. EPA also was reluctant to adopt a test that gave no direct measurement of the minimum lethal dose for a substance.

But these differences now seem to have been ironed out, after a meeting in Washington late last month. As a compromise, OECD's chemicals division has agreed to recommend that a preliminary 'sighting study' be carried out before each fixed-

dose test. The idea is to test single rats (probably females, which are generally more sensitive to poisoning) with varying doses of the chemical, until a dose is found that will just kill. This would allow US toxicologists to collect some data on the minimum lethal dose of a chemical, albeit of dubious statistical relevance.

The sighting study may also allow the Americans and Japanese to test toxicity at doses other than the four standards chosen for the main fixed-dose trials — which would help in applying the fixed-dose data to the US and Japanese systems for classifying dangerous chemicals.

The OECD's policy-making council is due to consider the changes at a meeting in Paris on 24 November, and Hugo van Looy, from OECD's chemicals division, says "the prospects are good" for the new recommendations to be accepted. Once the OECD changes its guidelines on toxicity testing, the European Commission, which has been considering a similar change for some time (*Nature* **341**, 680; 1989) is expected to rapidly follow suit.

**Peter Aldhous**

## SPECIES REINTRODUCTION

# New twist in snail saga

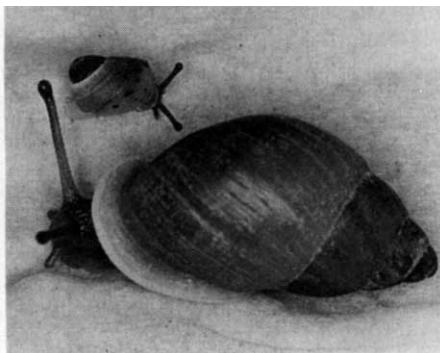
PREDATORY snails still stalk the tiny South Pacific island of Moorea three decades after a well-intentioned effort at biological control went disastrously wrong, researchers returning from a expedition to the island said last week. But the appetite of the rampaging human-introduced *Euglandina* snails for snails of other species on the island may soon be their undoing. In less than a decade, says expedition member Roger Klocek of Chicago's John Shedd Aquarium, "they will have eaten their way through all the other snails on

*Partula*. Among the various barricades that are being discussed to keep out the marauding *Euglandina* are screens, copper sheets, and electric fences. A year-round caretaker would have to watch the ramparts for breaches.

The tale of how the snail predators came to dominate the island is virtually a case study in bad biology. Island farmers brought Giant African land snails to Moorea in the mid-1960s as a food source. But the snails soon escaped from their farms and began to devour local crops. In 1977, local authorities, hoping to control the land snails, imported *Euglandina*. The predatory gastropods found the half-inch *Partula* more enticing, however, wiping them out in a decade.

*Partula* now exists only in some 3,000 snails of six species in a few breeding colonies around the world. And even those are proving hard to maintain, as some colonies inexplicably 'crash' from hundreds of snails to just a few individuals. In their research on a small nearby island where some *Partula* still remain in the wild, expedition team members may have found an explanation. Dead leaves and high humidity turn out to be more important to *Partula* diet and environment than they had realized, they say. Eating their own faeces — or "reingesting their gut flora", as the biologists put it — also appears to be more important to *Partula* digestion than thought. "We may have been keeping their cages too clean," says team leader Dave Clarke of the London Zoo.

**Christopher Anderson**



It's a snail-eat-snail world.

the island" and will probably die off. Then, the researchers hope, they can safely reintroduce the once-thriving *Partula* tree snails that *Euglandina* drove to extinction on Moorea in 1987.

In the meantime, the French Antenne Museum, which already has a research station on the island, is considering setting up a fenced-in, one-acre compound in which to start a trial reintroduction of

# Yellow light on L-tryptophan

MORE than two years after batches of a genetically engineered amino-acid food supplement were first implicated in 27 deaths from a rare blood disorder, the jury is still out over whether recombinant-DNA technology can be given any blame for the incident. But new data from animal experiments suggest that L-tryptophan in any form — not just the batches produced by the Japanese company Showa Denko that were linked to the deaths — may be inherently dangerous. The new results are likely to put renewed pressure on the US Food and Drug Administration (FDA) to regulate amino-acid food supplements as drugs, as the agency reviews its policy on the issue.

Opponents of genetic engineering, led by Jeremy Rifkin of the Foundation on Economic Trends, made the Showa Denko incident a *cause célèbre* once it emerged that the 1989 deaths from eosinophilia-myalgia syndrome (EMS) were associated with particular batches of L-tryptophan made by the Japanese company after it had introduced a new genetically engineered bacterial strain to produce the amino acid (*Nature* **346**, 787; 1990). Rifkin argued that the FDA should stop licensing new genetically engineered products until there had been a full inquiry into the deaths and a thorough review of the agency's regulation of biotechnology products. Late last month, the FDA denied Rifkin's petition, concluding that biotechnology offers no unique hazards and thus should not be regulated any differently than other methods of producing drugs.

For two years, researchers at Showa Denko and many laboratories in the United States have been struggling to discover just what caused the 1989 tragedy. Suspicion has focused on a compound called 1-1'-ethylidenebis[tryptophan], or EBT, which was present in far greater quantities in the case-linked L-tryptophan than in earlier batches produced by Showa Denko. EBT is a dimer consisting of two L-tryptophan molecules, produced by the reaction of the amino acid with acetaldehyde. According to Showa Denko, EBT did not seem to be present in fermentation broth containing the genetically engineered *Bacillus amyloliquefaciens*, but instead appeared later in the production process, during the purification of L-tryptophan. Showa Denko researchers have also failed to find any difference in the amounts of acetaldehyde found in fermentation broths containing their old bacterial strain and those with the new genetically engineered strain at which Rifkin had pointed the finger of blame.

Because Showa Denko reduced the amount of powdered carbon in its purification system at the same time as it introduced the new genetically engineered bac-

terial strain, these findings would seem to absolve genetic engineering from blame — provided that it was EBT that caused the EMS outbreak.

The new results, which will be presented at the American College of Rheumatology's annual meeting in Boston next month, are from a study designed to discover if EBT could have caused the EMS cases (which affected more than 1,500 Americans in addition to the 27 who died). Lori Love, an FDA scientist working with National Institute of Mental Health researchers, says that her team's results do indicate that EBT was involved in the EMS outbreak: rats treated for six weeks with the EMS-associated batches of L-tryptophan, and those treated with EBT alone, develop pathologic changes similar to those seen in EMS.

But Love and her colleagues have also found that rats treated with batches of L-tryptophan not linked with the EMS cases — and not produced with genetically engineered bacteria — developed similar symptoms, though in a less severe form. According to Love, the new study casts doubt on the safety of consuming large quantities of L-tryptophan. The data support previous studies, she says, that postulated a role for L-tryptophan in illnesses

that produce fibrous tissue in the skin, muscles and the body's organs.

Robert Hill, from the Centers for Disease Control in Atlanta, who collaborated on the study, points out that, in addition to EBT, a number of other compounds — which have not yet been identified — also seem to be associated with the EMS cases. Until these compounds are isolated and tested in the animal model, it will not be possible to determine for certain whether or not these other compounds are implicated in EMS. The data to date indicate that there is no role of genetic engineering in the formation of EBT.

The new results come just as FDA is reconsidering its policy on amino-acid food supplements. At present, FDA does not require amino acids to be regulated as drugs. But some amino acids are known to be metabolically active. L-tryptophan, for instance, is metabolized to the neurotransmitter serotonin and was taken by many of the EMS victims to relieve depression, insomnia or other nervous disorders. The suggestion that taking any L-tryptophan preparation could increase the risk of developing EMS will add to the pressure — voiced at a congressional hearing during the summer — on FDA to regulate the US market in amino-acid food supplements. Neighbouring Canada already treats these products as drugs.

**Peter Aldhous**

## AUSTRALIA

# Costly commercialization

## Melbourne

EFFORTS to commercialize the research activities of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) have cost the agency dearly, a government audit has found. The Australian National Audit Office estimates that CSIRO, Australia's biggest science agency, today spends most of its annual budget on outside research projects and says commercial research is being funded at the expense of basic science.

In 1988, the government directed CSIRO to obtain 30 per cent of its funding from external sources, which it has finally achieved this year. By doing so, however, it has diverted much of its resources and core activity into supporting industry-based research projects or attracting external funds from competitive grant schemes. "As a result, CSIRO is at risk of losing full control of its research and development programme," the auditors concluded. The report, which is critical of CSIRO's business dealings and management practices, says CSIRO personnel are negotiating commercial deals with an inadequate grasp of how much any agreed research and development (R&D) project will cost CSIRO. In particular, it found that undervaluing its contributions to some joint

ventures has led to cases where CSIRO's equity was "not proportionate" with its investment.

In the past three years, CSIRO has moved heavily into external funding arrangements, joint ventures and commercialization deals with private industry. By 1990, it was receiving \$167 million (about 26 per cent of its income) from non-budgetary sources, chiefly research corporations in the meat, grain and wool industries which traditionally have obtained CSIRO's intellectual property at well below full cost. CSIRO also holds equity in 12 private companies, with interests ranging from metals processing to aviation simulation.

CSIRO has shrugged off the report, claiming it says nothing new about the effects of the 30 per cent external funding target. Chief executive John Stocker has acknowledged that some early agreements were poor, but says CSIRO is pushing the federal government for policy changes that would force the rural industries and others to pay more for their CSIRO-derived R&D. "The [auditors] would have done better to support CSIRO's advocacy of these policy changes to the Government rather than imply deficiency on CSIRO's part," he said. **Brett Wright**



## Science university debuts

### Hong Kong

THE Hong Kong University of Science and Technology, the British territory's third fully fledged university and the first devoted specifically to the study of scientific topics, opened its doors last week. The school is expected to play a significant role in the economic transition of Hong Kong "from a low-tech, labour-intensive economy to a high-tech information-based society", in the words of the university vice chancellor, Woo Chia-wei.

With a population approaching 6 million squeezed into a space of scarcely more than ten hectares with few natural resources, Hong Kong has had to live on its wits. Over the past three decades, it has done so in a low-technology way, using cheap labour to manufacture electronic components, assemble consumer electronic devices and manufacture garments.

Now, however, the situation is changing. Asian nations such as Malaysia and Thailand are moving in on Hong Kong's low-technology markets, while the other 'little dragons' — Singapore, South Korea and Taiwan — are moving on to more sophisticated forms of technology. To survive, Hong Kong's companies must upgrade their technology.

The government already pays for research and development at two public universities and two polytechnics, but the universities are too diverse and the polytechnics not well enough accepted academically to provide leadership in Hong Kong's world of technology. The new university promises to fill the vacuum.

It will do so in large measure by encouraging postgraduates, who now make up fewer than 10 per cent of students at Hong Kong's universities. The new uni-

versity has about 140 postgraduates in its first group of 700 students, and it plans to increase the number to 30 per cent.

The emphasis on postgraduate education should help Hong Kong's economy in two ways. First, it will provide a local alternative for talented science graduates who in the past have felt forced to travel overseas to continue their studies. Too often they never return. Postgraduate studies will also provide a link to the local industrial community through joint research and development projects.

Located in a seaside setting in Hong Kong's New Territories, the new university has four schools: science, engineering, business and management, and humanities and social sciences. Students in science and engineering can read for undergraduate and higher degrees in biochemistry, biology, chemistry, mathematics, physics, computer science, and electrical and electronic engineering. Postgraduate studies in mechanical engineering and civil and structural engineering are now available; those subjects will be extended to undergraduates next year, and chemical engineering and industrial and mechanical engineering will be added in 1993.

The university, which can now accommodate 2,000 full-time students, intends to enrol 1,000 undergraduates and 250 graduate students in its second academic year, beginning in October 1992. A second phase of construction, due to be completed in 1993, will permit a student population of about 7,000 by the 1993-94 school year, while a third phase will bring the institution up to its planned capacity of 10,000 students by 1995-96.

Peter Gwynne

### JAPANESE RESEARCH CITIES

## Glaxo and TI open in Tsukuba

### Tokyo

JAPAN'S Tsukuba science city seems to be becoming increasingly attractive to some of the world's top research-oriented companies. Within days of each other, the UK pharmaceutical giant Glaxo and the US semiconductor manufacturer Texas Instruments (TI) opened new basic research institutes in Tsukuba. They join a small but growing list of well-known foreign companies based in the rapidly expanding science city.

The five-story Glaxo institute, which opened 26 September, was built at a cost of ¥20,000 million (\$150 million). It will have an initial complement of 110 staff, rising to 250 by 1996, and will focus on research and development of drugs in the fields of cancer, immunology and inflammatory diseases. Glaxo joins many Japa-

nese pharmaceutical companies already based in Tsukuba, as well as Upjohn of the United States, which opened a ¥14,000-million laboratory with 170 researchers in 1988, and ICI of the United Kingdom.

TI's new research and development centre opened five days after Glaxo's. The ¥5,400-million centre is expected to have about 100 researchers when recruitment is complete. The US semiconductor manufacturer Intel also has an institute in Tsukuba. Intel's laboratory concentrates on development of products fairly close to market, but the TI institute — like the fundamental NEC research institute next door — will concentrate on more basic research and in particular on nanotechnology, a topic that is rapidly catching the interest of Japan's industrial research community.

David Swinbanks

## Columbus delayed

### London

ALTHOUGH the US Congress has agreed to grant the full 1992 presidential budget request for the space station Freedom (*Nature* 353, 372; 3 October 1991), the controversial international project now faces another budgetary hiccup. Next month, ministers from the 13 nations of the European Space Agency (ESA) are expected to give their continued backing to the Columbus project — which includes ESA's contribution to the station — only if elements of the project are delayed in order to reduce costs over the next few years.

One such element will be a free-flying unmanned microgravity laboratory designed to orbit with the station. Fredrik Engstrom, who is directing ESA's contribution to the space station, says the launch of the free flyer will be delayed by two years, to 2003. (When first conceived, it was to have been launched in 1999, but previous delays have already shifted this to 2001.) Engstrom says there will now be no "hard-core development work" on the free flyer for the next three years. The precise amount of money saved by the delay has not been calculated, he says, but one thing is sure: despite the short-term savings, any launch delay will increase the total cost of the Columbus project.

ESA's manned laboratory, which will be bolted onto the main station, should still be launched in 1998, as planned. P.A.

### BIOMEDICAL RESEARCH

## Rockefeller loses one

### Washington

NOBEL laureate Gerald Edelman and the Neurosciences Institute that he directs will leave Rockefeller University for greener pastures at the Scripps Research Institute, Edelman announced early this week. Scripps offered the Institute a new \$7-million building, something Edelman describes as a "magnificent opportunity" that he could not turn down. Critics of Rockefeller president David Baltimore note that Edelman is the second prominent Rockefeller scientist to leave in as many months, following diabetes researcher Anthony Cerami (see *Nature* 352, 463; 8 August 1991). But Edelman plays down speculation of a link. "The old Rockefeller system was one I very much admired," he says. "It has changed since then, but that in no way influenced my decision." Scripps, meanwhile, can add Edelman to its quickly growing list of notable recruits to the southern California research campus. With funding that has more than doubled in the last five years to a total of \$85 million, Scripps has in the last year snared such prominent researchers as chemists Barry Sharpless and K.C. Nicolaou, molecular geneticist Sydney Brenner and plant biologist Roger Beachy. C.A.

# Penicillin and beyond

Benjamin Chain

The discovery of penicillin remains one of the greatest advances in medical science. From the success of the discovery the biotechnology industry became established.

THE occasion of a fiftieth anniversary is one for retrospection, and half a century of penicillin therapy has been no exception. The story of penicillin is better known and more publicized than almost any comparable event in the history of medical science. The anniversary is indeed a time of celebration, for the achievements of penicillin chemotherapy have exceeded even the most optimistic expectations, and remain one of the biggest advances in medicine ever made.

Three researchers were recognized, by the award of the Nobel prize for medicine in 1945, for their central role in the discovery of penicillin. By this time, Alexander Fleming, whose celebrated chance observation of penicillin's antibacterial activity in 1928 laid the foundation for the later studies, was already reaching the end of his career. Howard Florey, the head of the Oxford department in which penicillin was first tested and purified, went on to become president of the Royal Society and a leading figure in the post-war British scientific establishment. But from the vantage point of 1991, nowhere is the foreshadowing of the contemporary era of the 'new' biotechnology more dramatically illustrated than in the post-war career and achievements of the youngest of the three major penicillin protagonists, Ernst Chain. In this article I discuss how Chain built on the success of the penicillin discovery to found two unique institutes of biochemistry, which significantly influenced the further development of the particular branches of science in which he was involved, and especially fostered the subsequent expansion of the biotechnology industry. In establishing these institutes, Chain explored the path of academic industrial collaborations, interdisciplinary research centres and scientific internationalism 20, 30 or even 40 years before these concepts became the buzz-words of the contemporary scientific establishment.

Chain initiated the Oxford phase of the penicillin discovery, when he undertook, for purely theoretical reasons, to re-examine Fleming's initial observations on the antibacterial activity produced by the *Penicillium* mould. Having convinced himself, and Florey, that even in crude preparations penicillin had astonishing antibacterial power *in vivo*, as well as *in vitro*, Chain, together with his Oxford colleagues, undertook the purification and characterization of

penicillin, and the production of sufficient partially purified material for the first clinical trials. These studies achieved rapid and sensational success. But soon after 1945 Chain left Oxford, increasingly frustrated by his failure to obtain financial support on a scale that would enable him to compete with the US research effort in developing the antibiotic field, and by what he perceived as an environment that fostered antiquated, damaging divisions between academic institutions and industry.

Chain's post-Oxford career was embodied in the establishment of two major centres of biochemical research, one in the Institute of Public Health laboratories in Rome (in which he worked from 1949 to 1962), and the other at Imperial College London (which opened in 1965 and in which he remained until his retirement). The selection of these two sites was no coincidence. Both were situated in capital cities of international attraction and prestige for past as much as present achievements — these pro-

outside the conventional university framework, the Rome institute because of its special relationship to central government, and Imperial College because of its strong tradition in promoting the technological sciences. These circumstances lent an independence of spirit to both institutions, enhanced by the exceptional characters of their respective directors at the time, Domenico Marotta and Patrick Linstead, and allowed them to adapt more readily to Chain's unusual blend of pure and applied science which had met with little favour within the more traditional academic environment of Oxford. In addition, combinations of circumstances allowed both institutes to provide financial backing on an almost unprecedented scale for an academic department of biological sciences — this was essential in building a unique feature of both departments, an industrial-scale fermentation pilot plant containing reaction vessels with individual capacities of more than 3,000 litres. Chain's inability to attract funding in Britain, from gov-



Ernst Chain — breaking down traditional boundaries.

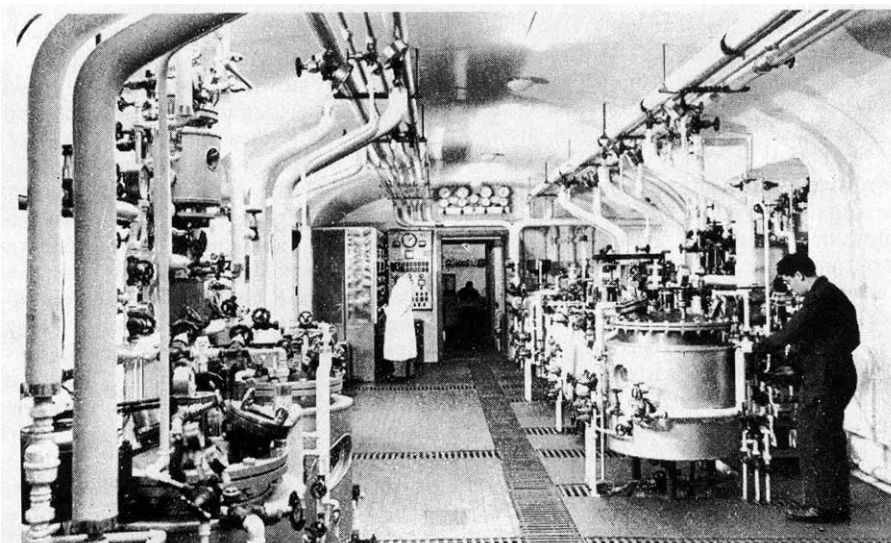
vided an appropriate environment for the aspirations of both laboratories to become international attractions and centres of excellence in their own right. The broader cultural appeal of both cities, reminiscent of Chain's own native pre-war Berlin, was certainly a determining force in bringing not only Chain himself but an uninterrupted flow of eminent scientific visitors. These visits stimulated a highly productive cross-fertilization of ideas, playing a major part in creating the intellectual environment of both departments.

Both institutions stood somewhat

ernment or industry, for such a venture was an important reason for his departure from Oxford, and these pilot plants were at the very heart of his ambitions.

Influenced by his experiences during the first frustrating attempts at scaling-up penicillin production in the Oxford laboratories using antiquated and inappropriate technologies, Chain was convinced that progress in isolation and characterization of biologically active substances (not only antibiotics, but vitamins, hormones, growth factors and other biological molecules active at very low concentrations) absolutely required





The fermentation plant at the Rome laboratories.

large-scale production of biological material. In the case of microbial biochemistry, Chain's own special interest, this meant large-scale fermentation facilities. A pilot plant on a semi-industrial scale was therefore not a luxury, nor was its purpose to carry out routine contract research for industry, work which Chain strongly believed could be carried out much better in industrial laboratories. Such a pilot plant was simply an essential piece of equipment, which differed in size and cost, but not in its essential purpose, from the routine culture facilities of the microbiological laboratory, and which could therefore be justified simply by its value to scientific progress.

Both in Rome and later in London, Chain's ambitions to work on a scale unprecedented within an academic biochemistry department were fulfilled. In a letter to a colleague in 1949, Chain comments: "The Institute in which I am working is exceptionally well-equipped, in fact almost too luxuriously. I am not used to this type of laboratory . . ." — this after Oxford University, and in an unknown institute in Italy when that country was barely recovering from wartime austerities.

More than anything, both in terms of physical design and in terms of function, the London and Rome institutes symbolized the destruction of traditional boundaries in more than one sense. Boundaries between disciplines were removed in that Chain's two main interests, microbial biochemistry and mammalian intermediary metabolism, required knowledge of biochemistry, microbiology, organic chemistry, engineering and electronics, and demanded integration of these disparate subjects. As Chain often emphasized, this interdisciplinary nature was an essential and fundamental feature of the organization of his departments. Boundaries between academic institutions and industry were broken down, not just in a sense that industry provided

financial backing for academic projects, but much more intimately in providing an environment where problems of academic interest and practical interest co-existed, and where both projects and personnel frequently switched from industrial laboratories to the academic departments, and back again. Finally, national boundaries were removed, for both the London and Rome laboratories became international centres attracting and training scientists from many developed and developing countries. The Rome institute, for example, was nominated almost from its inception as a World Health Organisation centre for training in microbiology and fermentation technology, a role which it continued successfully for many years.

In every one of these characteristics, the development of the two institutes closely mirrored Chain's personal interests, ambitions and goals. In terms of internationalism, Chain by background and temperament was a cosmopolitan. Long before it became fashionable, he was a European, convinced of the importance of collaboration between countries so that Europe could compete with science in the United States. Chain was also unusually successful in bridging the gap between academic institutions and industry, although he was not reluctant to criticize industry when, in his opinion, it failed to invest adequately in research enterprises. In valuing the industrial contribution, he may have been influenced by his family background (his father had taken a doctoral degree in chemistry before establishing a company making copper sulphate and related compounds outside Berlin before the First World War), as well as the general atmosphere in pre-war Germany where interaction between academic institutions and commerce was valued and highly productive. In Chain's case, he was a consultant in the development of several companies, including the phar-

maceutical arm of Beechams, the Swedish pharmaceutical company Astra, and the food technology section of Rank Hovis and McDougall. He seemed to be untroubled by the potential conflict of loyalties between the goals of academic institutions and private industry, and felt that such consultancies were "good for the professor, the firms and most important of all, for the productivity of the country". There can be little doubt that this close involvement with the pharmaceutical industry, and particularly the financial benefit he derived from it, was regarded with deep suspicion, if not jealousy, by many in the scientific community.

Chain's own career also predisposed him to an interdisciplinary approach to scientific problems. He trained as an organic chemist, turned later to biochemistry, and ultimately became interested in bioengineering. It was his ability to communicate with engineers, organic chemists and biologists of different disciplines that was perhaps his most impressive quality, and that enabled him to mould the integrated multidisciplinary teams that formed the core of both the Rome and London departments. What seemed to excite him was not so much the ultimate significance of a problem, in terms of the progress of biochemistry, but the explanation of unusual biological phenomena, and particularly those whose solution involved a technical biochemical challenge. As he himself recollected in later years, it was not the thought that penicillin would be useful that stimulated the start of the Oxford investigations, but simply that the material had potent biological activity, and yet was unstable and difficult to isolate by conventional chemical techniques.

A look at the publication lists of the two institutes reveals an astonishing combination of papers devoted to fundamental questions of biochemistry (the mode of action of insulin, or the principles of fungal genetics) alongside new designs for filtration equipment for large submerged culture fermenters, new apparatus for continuous measurement of dissolved oxygen during fermentation, new quantitative scanners for two-dimensional radioactive chromatography for the study of intermediary metabolism, and many other similar contributions of an essentially technological nature. It was Chain's refusal to dismiss a problem because it was "merely practical", but rather his insistence on applying a strict scientific approach to just such problems, that allowed his institutes to make some of their most important contribution to their fields, and that most cemented the close links between the academic and industrial worlds. In addition, it was this approach that engendered in both institutes the environ-

ment in which dozens of scientists from many countries were trained and influenced. Many of these 'human products' of the institutes remained in academic departments but, characteristically, many then went to work in industrial research and development laboratories, making contributions to the post-war growth of the biotechnologically based chemotherapeutic and food industries.

The discovery that most typified Chain's approach, and had the greatest immediate practical impact, was the development of the first generation of semi-synthetic penicillins. At Chain's suggestion, Beechams had begun a collaborative study with the Rome institute to produce chemically modified penicillin molecules with improved antibacterial properties. As part of these studies, Beechams sent some researchers to Rome to learn the latest technologies in penicillin production. During this time, the pilot plant personnel performed some by then standard 'runs' for the production of penicillin, during which multiple parameters (aeration efficiency, power consumption, mycelial growth characteristics and so on) were carefully monitored and compared to penicillin yields measured in terms of biological activity and chemical analysis.

On reviewing these data, Chain and the Beechams' researchers were struck by an apparent anomaly between penicillin yield as measured by chemical and biological assay. This apparent experimental inconsistency was followed up, leading directly to the discovery that, under appropriate conditions, the *Penicillium* mould can produce 6-amino peni-

ited Kingdom the lead in the penicillin field that had been lost to the United States soon after the original discovery.

Despite his scientific successes, Chain's career, from the time he arrived in England in 1933, was dogged by controversy, arising in part from his personality — he was always outspoken, supremely confident in his own judgement, impatient of delay or bureaucracy, and he pursued his objectives with an unflagging enthusiasm that struck many as obsessive. He left his first post (at University College Hospital, London, under Charles Harington) within a few months, appalled at what he considered to be the low level of facilities in that laboratory. He left Oxford after the war, having quarrelled with the Medical Research Council over its refusal to patent the penicillin discovery, disgusted with both government and industry over their unwillingness to provide large research funds for a fermentation pilot plant. On his return to Imperial College, he clashed repeatedly, often acrimoniously, with funding bodies over their policy in relation to biochemistry in general, and his own department in particular.

Finally, his retirement was marred by an extraordinary and lengthy battle with the then rector of Imperial College, Lord Flowers, over the appointment of his successor. Chain was extremely concerned that the two branches of biochemistry in which he was most interested, microbial biochemistry and the study of metabolism at the organ/whole-body level, should be maintained at the Imperial College department, and not be replaced by molecular biology, a discipline that Chain considered already to be over-represented in the United Kingdom. To this end, he persuaded the Rank Foundation to donate a second chair of biochemistry to the department to be known as the Rank Chair of Physiological Biochemistry. Inevitably, this attempt to influence the future development of his department was doomed to failure, and after Chain's retirement, the Imperial College biochemistry department became one of the leading molecular biology centres in the country. Microbial biochemistry, and, in parallel, the fermentation pilot plant suffered a continuous decline. It is significant that the Science and Engineering Research Council recently chose University College, not Imperial College, as the site of its Interdisciplinary Research Centre in bioengineering, the plans for which include a fermentation pilot plant.

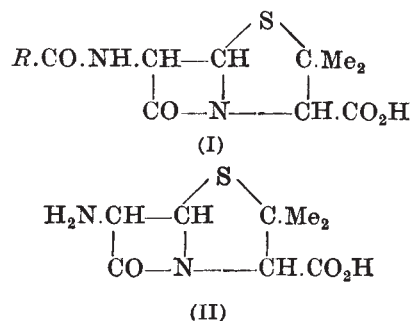
There can be little doubt that Chain underestimated the future contribution of the molecular biological revolution to biochemistry. Equally, it remains true that today, 20 years after his retirement, the contributions of molecular biology to medicine, compared with conventional

drug development, have remained small.

Many of Chain's views are gaining favour only now. In words, if not yet in deed, the academic establishment and government bodies involved in science funding in Britain are trying to break down boundaries in the way that Chain's type of science foreshadowed. Biotechnology and commercial exploitation of scientific invention are no longer despised, but are almost slavishly sought-after goals. But the nearly 50-year delay between Chain's espousal of these causes and their implementation has exacted heavy costs. It is depressing to note the parallels between British scientific achievement in the biochemical fields as reviewed by Chain in the early 1960s, and the situation now. Chain's often repeated complaint at that time was that antiquated attitudes on the part of both government and industry were responsible for the fact that the isolation and structural characterization of virtually every growth factor and vitamin known had taken place outside the United Kingdom, despite the fundamental role of British science in the work that had led up to their discovery. With hindsight, this seems a remarkable anticipation of a current parallel situation in regard to the whole array of growth factors and cytokines uncovered by recombinant gene technologies during the 1980s, hardly one of which was isolated or biochemically characterized in Britain.

Even today, despite the rhetoric of the past ten years, the breakdown of traditional boundaries within the biological sciences, especially in Britain, seems to be only just beginning. The essential message of Chain's career remains that superficial attempts to induce interdisciplinary approaches, and to foster industrial-academic collaboration, are simply not enough. Only where commerce and academic institutions develop a genuine symbiosis, so that they can really come to understand each other's strengths and weaknesses, can one hope to achieve really productive results. Chain's words, written in 1962, still seem apposite: "Altogether, I am convinced that the movement towards breaking down unnecessary and antiquated barriers and prejudices will do only good, for both science and industry. We need the closest collaboration between the two." □

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6-amino penicillamic acid.

cillamic acid, the chemical nucleus of the penicillin molecule, which on its own has no biological activity, but which provides a versatile and cheap starting point for the chemical production of penicillin analogues, some of which have greatly enhanced antibacterial activity and did not have many of the problems of penicillin resistance. Within months, the combined efforts of the Rome and Beechams' establishments had turned an apparently insignificant experimental anomaly into an enormous commercial enterprise, which recaptured for the Un-

I thank the Contemporary Medical Archives Centre at the Wellcome Institute Museum of the History of Medicine for permission to consult their collection of the letters and papers of Sir Ernst Chain, and Mrs J. Alton of the Contemporary Scientific Archives Centre in Oxford for her work in supervising the organization and cataloguing of this collection.



# Baltimore's unanswered questions

SIR — Rather than replying in detail to David Baltimore's open letter to me (*Nature* 353, 9; 1991), I would suggest that interested readers compare his letter with my Commentary article (*Nature* 352, 183; 1991). The disconnection is nearly total. I stated: "Reviewing the case strictly from Baltimore's published account reveals at least four lapses . . .". Baltimore counters with: ". . . your verdicts are . . . based mainly on the unsubstantiated, and often refuted, allegations of one participant . . .". My statement is true, his is not. In passing, it is interesting to note Baltimore's changing views of the "one participant, Dr O'Toole". Only four months ago he wrote of her: "I have tremendous respect for Dr O'Toole . . . and I believe that her expressions of concern were proper and appropriate, and her motives were pure" (*Nature* 351, 94; 1991).

Here is another example. I stated that "until the final OSI report is released, we will not know the extent to which the opposing views of the authors of the *Cell* paper will have affected final judgements". Therefore I did not use any of the findings of the draft report in my argument. Yet Baltimore rebukes me for using "a leaked, confidential draft of a government report as a basis for definitive judgements about the acts of others".

Against my claim that his own testimony in this case shows a regrettable departure from normal standards, he replies that: "I believe that my science — including the Weaver *et al.* paper — is done with vigour and criticality" — a claim that stands in contradiction to his retraction of the paper. One can judge which of these views is more nearly correct by noting Ptashne's demonstration (see below) that essential data in the Weaver *et al.* paper is either nonexistent or undecipherable. Baltimore then goes on to say that the only way to judge his paper is to demonstrate whether or not the data are reliable. But the presentation of the 'data' is such as to prevent the application of this test.

The claim is then made that "much published evidence . . . support[s] his paper's results in remarkable detail". However, an examination of this evidence shows that it is not at all evident that the six papers referenced provide any support at all. But there is a deeper criticism of Baltimore's claim that all is well if his conclusions are ultimately shown to be correct. The scientific literature would become irredeemably corrupted if this became accepted practice. The essential standard is that the evidence presented in a scientific paper is the bedrock on which interpretations

and conclusions are built. If this connection is violated so that speculations drawn depend on subsequent investigations to prove right or wrong, then the reporting of research would be reduced to a lottery.

The missing RNA band is dismissed with "its existence was not of significance to the paper". However, if the band was visible on the gel, were the authors not obliged to state this in the paper and comment on its significance? If not, does this not lower existing standards by affirming that unwelcome data need not be presented or mentioned?

The part of my Commentary concerned with Baltimore dealt almost entirely with criticizing his behaviour and urging that it should not contribute to debasing past standards of conducting and reporting research. In his reply to me, Baltimore ignores this central theme and insists that he has always abided by the higher standards. This is the ultimate disconnection: alas it shows no sign of being bridged.

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SIR — In his response to my letter of 11 July (*Nature* 352, 101; 1991), Erik Selsing (*Nature* 352, 657; 1991) says the "central claim" of Weaver *et al.* (*Cell* 45, 247–259; 1986) is "most directly" supported by data describing idiotype-positive antibodies whose heavy chains are  $\gamma$  and  $\alpha$  (as distinguished from  $\mu$ , the isotype of the immunoglobulin M (IgM) transgene-encoded antibody). Indeed, the presence of such molecules significantly above the level found in control animals would support the central claim, provided they were not encoded by recombinants formed between the transgene and endogenous genes. But where are these essential data?

Initially, referring to Table 2 of their paper, Weaver *et al.* wrote on page 250 that: "Of the 172 idiotype-positive hybridomas, only 53 were IgM secretors. The remaining 119 clones produced other Ig heavy chain isotypes, the majority being  $\gamma_{2B}$  (data not shown)." Then, in a letter of correction (*Cell* 55, 541; 1988), the authors said: "Again relating to Table 2, the non-IgM-containing wells were referred to as 'mostly  $\gamma_{2B}$ ' in the text. In fact, the data showing that a majority were  $\gamma$  (not necessarily  $\gamma_{2B}$ ) came from separate experiments, not the ones depicted in Table 2."

Although the authors themselves do not say to which experiments they are referring, Selsing says that the crucial isotyping results are presented in Table 3. This table, both in the original and in the version published in a further correc-

tion (*Cell* 57, 515; 1989), lists 34 hybridomas and states, for 30 of these, whether the secreted antibody heavy chain is  $\gamma$ ,  $\alpha$  or  $\mu$ ; four are listed as not determined. Fifteen are claimed to have  $\gamma$  heavy chains — but 15 is hardly a "majority of" the 119 mentioned in the original text. One is left wondering whether Table 3 does in fact contain the isotyping results referred to in the correction of 1988.

Nevertheless, as suggested by Selsing, perhaps the crucial isotyping data are contained in Table 3; if so, we are faced with the problem that the paper does not tell what experiment was actually done. For example, are all the listed hybridomas idiotype-positive? Which of the two assays that detect idiotype-positive molecules, described in the methods section, was used — or were different methods used for different entries? Are we to assume that each entry corresponds to a cloned hybridoma producing a single species of antibody and, if so, where is the evidence? Where are the corresponding results for control mice?

At the time its draft report was written, the Office of Scientific Integrity evidently was not able to resolve ambiguities in Table 3 despite (presumably) a far more extensive analysis. Referring to the results of isotype assays (ELISA) reported in Table 3, the draft report (p. 110) says: "It appears that the choice is between believing the serological methods employed were seriously inadequate or the ELISA was mislabelled and misrepresented."

We are considering the crucial experiment, reported more than five years ago — and challenged soon thereafter — followed by two published corrections. One would have thought there had been ample opportunity for the authors to have clarified the issues. If Selsing has satisfactory answers to these essential questions, why aren't they available to the rest of us?

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## Energy costs

SIR — You fear that rising energy prices would hurt the economy (*Nature* 352, 649, 1991). A look at the facts may be useful. Among the countries with the biggest industrial output energy prices from 1975 to 1990 were highest in Japan, followed by (West) Germany, for United States and finally by the Soviet Union all with a wide margin.

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## Unspoken fears

SIR — With regard to safety provisions for environmental releases, organisms genetically modified by recombinant DNA techniques are often singled out as intrinsically risk-entailing. This widespread attitude leads to scientifically untenable distinctions, such as the inclusion of site-directed mutagenesis among the techniques to be kept under scrutiny, and the simultaneous exemption of random mutagenesis. By equating 'natural' with 'safe', this prejudice grossly underestimates the risks posed by incautious environmental introductions of 'natural' organisms. The most dramatic predictions made for recombinant organisms apply equally to conventional ones.

Excessive proliferation of conventional organisms in the wake of their 'planned' introduction (rabbits in Australia, the Nile perch in Lake Victoria) has already caused ecological disasters. Mechanisms allowing horizontal gene transfer do not distinguish between recombinant and conventional organisms, and the spread of a natural, non-recombinant plasmid conferring resistance to an antibiotic in use represents a real danger, which should be rigorously kept in check. New, undesirable characters can emerge unpredictably as a result of conventional breeding<sup>1</sup>.

Such considerations reinforce the concept that, within the spectrum of practicable genetic modification techniques, the more precise the method of introducing a new trait, the lower the likelihood of an unpredicted effect. What generates confusion is the lack of accepted, general classes of environmental risk, similar to those applied in issues relating to human health. Leaving environmental risks in the realm of imagination continues to fuel the idea that environmental introductions of recombinant organisms entail unique risks.

I have drawn up a tentative classification of possible environmental risks, ranked according to level of concern (see table). The assignment of an organism,

### PROPOSED CLASSIFICATION OF ENVIRONMENTAL RISK GROUPS

- Organisms which were never recognized to pose a risk to the environment
- Organisms which may cause transient ecological imbalances or transient biogeochemical effects
- Organisms which might be pathogens or pests for plants or animals, but have limited diffusion or persistence
- Organisms which might transfer unwanted genetic traits to other species
- Organisms which may cause persistent undesirable ecological imbalances or persistent biogeochemical changes
- Dangerous and highly diffusive pathogens or pests, either for animals or for plants

whether natural or modified, to one of the risk classes would be based on the

properties of the organism and of the target environment. A careful description of the parental organism, of the traits added (or deleted) and of the nature and precision of the modification would appear among the attributes to be considered in assessing genetically modified organisms<sup>2</sup>.

Such a tentative list of possible environmental risks, which might easily be extended to include effects specifically attributable to recombinant DNA organisms, were any to be identified, was brought to the attention of the OECD group of national experts on safety in biotechnology at its plenary meeting in Paris on 26 June 1991. My approach was rejected, being labelled "premature".

May I ask whether it is still "premature", after so many years of debate, to try to state what are the real concerns? Or is a pyramid of power and regulation being built on unspoken fears?

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## Milky way

SIR — I have recently been in Nigeria, where an undereducated population is constantly exposed to advertising campaigns, notably by Nestlé, in which the full strength of current methods of persuasion is used to convey the seductive impression that formula feeding is the modern, westernized thing to do and will result in a bouncing healthy baby (and a happy smiling husband, too!). When the truth of these campaigns is apparently confirmed by gifts of free samples of formula from trusted clinics, mothers can hardly be said to be making a 'free choice' (*Nature* 352, 266; 1991).

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## Climate change

SIR — The contention of Jesse H. Ausubel (*Nature* 350, 649; 1991) that the world need not worry unduly about climate change because it has developed or can develop the technology for adaptation sounds rather hollow in developing countries. Those who have the money and the technology may be able to protect themselves against changes in climate. But has Ausubel given any thought to the vast majority of people on the globe who live in poverty? How would they be able to cope with the vagaries of climate change, which in the first place would be overwhelmingly a consequence of the technological

'advancement' of the rich nations? Even the rich countries may not be fully able to protect themselves against a very drastic change in climate.

Witness the recent havoc caused by a cyclonic storm in Bangladesh, where up to 300,000 people may have died, in spite of advance warning. Since then, Bangladesh has been battered by a series of storms in which several thousands have died. Here we are talking about 'normal weather' and not climate change as yet. What would happen if, as predicted by many models, there is an increase in freak storms? How would Bangladesh raise the resources to protect its vulnerable coastline and its people? How would the Maldives, a tiny nation of islands in the Indian Ocean, or other developing countries cope with rising sea levels caused by a warmer Earth? For that matter, can even a moderately rich nation such as the Netherlands save its precious land from the onslaught of the rising oceans if the increase in mean sea level is very high?

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## Watching the ball

SIR — With a background in physics as well as molecular biology, I believe I am a "qualified scientist" who has indeed observed ball lightning (*Nature* 350, 108; 1991). The following is a report I made immediately afterwards.

"During an incredible electrical storm [in Houston, Texas on 18 June 1991] in the evening while sitting at a table in the breakfast room, I saw a ball of lightning enter the utility room [an extension of the breakfast room] apparently through the back door. It hovered as a revolving sphere of bright yellow, orange and red light about 10 inches in diameter, in the air about three feet above the floor. It stayed in the same place. After about two or three seconds the globe disappeared with a loud pop rather like a discharge from a champagne bottle. This discharge was followed by a distinct odor of ozone. My Siamese cat also appeared to see the ball; at least he ran towards it."

This experience was amazing and interesting without being in any way frightening. My main thought was how much we scientists still do not know about natural phenomena.

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# Making the electron mass finite

A claim to have dealt with problems in quantum electrodynamics in such a way that the subtraction of infinite quantities from each other is avoided should lift the spirits of field theorists everywhere.

THE self-energy of the electron has been an embarrassment for almost a century, and in a sense for longer than there have been authentic electrons (first demonstrated by J. J. Thomson in 1901) and for far longer than physics has been complicated (but, some would say, simplified) by quantum mechanics. For if electrons are strictly point-like objects, their self-energy, most simply taken as their potential energy in the electric field which they carry, must be infinite.

People such as H. A. Lorentz were well aware of the difficulty. The simplest way of reaching this conclusion is to start with an electrified sphere and to calculate the potential energy of the charge distribution in the electric field which that same distribution generates. The result is a finite number. But then, if the radius of the sphere is allowed to shrink to nothing, the self-energy of the point-like charge becomes infinite.

In itself, that would be no great scandal, even if electrons were point-like entities, for the absolute value of a potential energy is unimportant. But by 1905, Einstein had sharpened the dilemma by demonstrating the equivalence of energy and mass. How, then, to escape from the conclusion that a point-like electron should have infinite mass?

The way out of that dilemma is to suppose that electrons are not point-like at all, but that they have a finite radius and that their self-energy is somehow contained in their measured mass. But, sadly, even that picture is unsatisfactory, for electrons in motion should then have the shape of ellipsoids, being contracted in dimension along the direction of their motion by the Lorentz-Fitzgerald contraction. (Fitzgerald, who taught at Trinity College, Dublin, at the turn of the century, seems to share the honour for that phenomenon only because Sir Oliver Lodge told a meeting of the British Association on Einstein's special relativity that "Fitzgerald has been teaching that to his students for years.") Only the arrival of quantum mechanics 20 years later sufficiently muddled the issue of the self-mass for it to be subsumed in grander questions.

Yet the embarrassment has been out in the open again for at least the past 40 years. It is an heroic tale. Feynman, Schwinger and Tomonaga were awarded a Nobel Prize in 1965 for their independent solution of the problem of the interaction between electrons (or, in principle, other

charged particles) with an electromagnetic field. The essence of the task was to find a relativistic solution of the problem first tackled by Lorentz half a century earlier, but taking full account of quantal character of the electron.

Each hero encountered the same difficulty — that the old infinities emerged again as stumbling-blocks. The infinite quantities tend to have the form of the integral of  $1/k$ , where  $k$  is the distance between some origin of coordinates and some variable point over the whole of three-dimensional or four-dimensional space. Plainly the quantities are strictly infinite, as is the integral of  $1/k$  in one dimension in the range from zero to infinity. (The indefinite integral is  $\log k$ , which tends to minus infinity as  $k$  tends to zero.)

So how to deal with these infinities? The trouble arises only when  $k$ , which stands for the wave number of electromagnetic waves, is very small. One way of accommodating the difficulty is to turn the integrals that become infinite into finite integrals by multiplying the integrand by a suitable factor whose effect will be negligible except when  $k$  is small. For practical purposes, this boils down to the subtraction of infinite quantities from the troublesome infinities. One triumph of Feynmann, Schwinger and Tomonaga is that they were able to do this consistently. Another, and the most striking, is that the results are astonishing in their accuracy.

Nevertheless, the practitioners of quantum electrodynamics have had to put up with a great deal of ribaldry for their practice of subtracting infinite quantities from each other. The procedure is hardly excused by the use of the term 'renormalization' to describe this systematic sleight of hand. And the circumstance that the calculations yield accurate predictions of measurable quantities is not much comfort to the purists.

That is why there may now be great rejoicing at the claim by R. Suzuki, from the University of Tokyo, to have discovered a way of carrying through calculations in quantum electrodynamics in a manner that avoids infinities altogether (*Il Nuovo Cimento* **104A**, 1,115; August 1991). Those who relish the details of the algebra, or who need to know them, will no doubt look them up. What follows is a description in words of what Suzuki is about.

First, because the objective is a relativistic

solution, there are four physical dimensions (one for time) and a metric (measuring the equivalent of distance) which is the sum of the squares of the three space coordinates minus the square of  $ct$ , where  $c$  stands for the velocity of light and  $t$  for time. Suzuki sets out to find a transformation of these coordinate axes that will make quantum electrodynamics manageable. He argues that a rotation in the sub-space formed by one space coordinate and the time coordinate will do the trick.

The procedure is then straightforward, but heavy, algebra. The quantum representation of the energy of a system of electrons in a radiation field, called the Hamiltonian, is simply written down in terms of the four-potential (the three-dimensional vector potential and the scalar electric potential) of the field and the four-current (the three components of the physical current and the charge density) that coexists with it. He then looks for (and finds) a unitary transformation (or a rotation in four-space) that splits the Hamiltonian into two parts, each of which is likely to give rise to infinities, but in such a way that the infinities cancel out.

This, according to the algebra, is indeed what happens. Suzuki calculates the self-mass of the electron, for example, and finds that the two separate parts of the Hamiltonian yield results that are equal in value but opposite in sign, at least in the approximation when the self-energy is expanded in powers of the square of the electric charge. And, moreover, by means of algebra so heavy that even the author is compelled to describe it in words, the same is true to the next approximation and, so, by induction, for all further approximations. For good measure, he also calculates the scattering of an electron in an electromagnetic field, again finding that the infinite quantities cancel out before it is necessary to calculate them.

There are two reasons why this development is important. The chief of them is psychological; it is awkward, to say the least, to have to live with theories in which infinite quantities are arbitrarily subtracted from each other. But quantum electrodynamics is not the only theory bedevilled by infinities, which crop up in most theories of particle fields. Suzuki says his colleagues have dealt successfully with several of them. What remains obscure is the physical meaning of the transformation (if any).

John Maddox

# Confirmation for quantum chromodynamics

Frank Close

THE missing link in the chain of proof for quantum chromodynamics (QCD) — the favoured theory of quarks, gluons and the interactions that build up protons and atomic nuclei — has been found at the Large Electron-Positron Collider (LEP) in Geneva. This news, announced earlier this year, has now been established in more quantitative and convincing detail, as reported at two European Physical Society meetings\*.

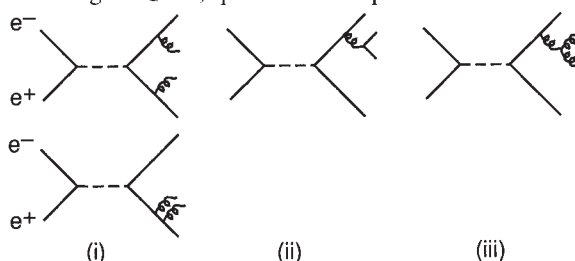
The results confirm that the strong forces underpinning the existence and interactions of nuclear particles are controlled by the same mathematical principles that govern quantum electrodynamics (QED), the well-proven relativistic quantum theory of charged particles and light. Although theorists, encouraged by the mathematical elegance of QCD and its similarity to QED, have believed this to be true for several years, and have already exploited this mathematical similarity to construct grand unified theories (GUTs), it is only now that this essential foundation has been proved.

Quantum chromodynamics and the road to GUTs grew from the discovery that protons, neutrons and all strongly interacting particles are built of quarks — fundamental particles with the same spin ( $\frac{1}{2}$ ) as electrons and which respond to the electromagnetic and weak interactions as do the electrons. One of the early proofs of this came in the annihilation of electrons ( $e^-$ ) and their positively charged antiparticles positrons ( $e^+$ ) after 1970.

The probability that an electron and positron annihilate and that their energy rematerializes as spin- $\frac{1}{2}$  particles, such as a quark and an antiquark ( $q$  and  $\bar{q}$ ) is calculable in QED. Moreover QED implies that the chance that the  $q$  and  $\bar{q}$  emerge at some angle  $\theta$  relative to the original  $e^-e^+$  beams (the 'angular distribution') is a sensitive measure of their spin. For example, spin- $\frac{1}{2}$  quarks are least likely to emerge perpendicular to the  $e^-e^+$  beams whereas particles without spin would prefer that direction. A problem is that individual quarks do not reach the huge detectors remote from the original production site; instead, sprays or 'jets' of high-momentum particles reveal the direction and momentum of the original quark and antiquark. The production rate and spatial distribution of these pairs of jets showed that their ancestors indeed have spin  $\frac{1}{2}$  as the

quark model requires.

The QCD theory of quarks was developed around the same time and implied that new, albeit rarer, phenomena should show up in the debris of  $e^-e^+$  annihilation. As accelerated electrons can radiate photons (according to QED and well-known experimentally) so, according to QCD, quarks or antiquarks



Electron-positron annihilation (at left) produces a  $Z^0$  (dotted lines) which converts initially into a quark and antiquark (solid lines). These radiate gluons (curly lines), ultimately producing four distinct quarks, antiquarks and gluons each of which generates a jet of hadrons in a detector.

should analogously radiate gluons. For every ten or so events with two sharp jets of particles, there should be one with three jets — two of these jets coming from quark and antiquark as before, the third coming from a gluon. The three jets would be clearly distinguished only when the gluon was radiated at a significant angle relative to the quark; at lesser angles the gluon and quark jets merge into an unresolved broad spray. According to QCD the gluons have spin 1 and, in turn, this leads to a characteristic angular distribution among the three jets.

These 'three-jet' events were clearly identified around 1980 by teams including one working at DESY in Hamburg. This was then the highest energy electron-positron facility in the world and provided enough energy that, even when shared among three jets, sufficient thrust was given to the particles for the individual jets to be resolved. Because 'three-jet' events are rarer than their 'two-jet' counterparts, it took longer to accumulate a sufficiently large sample of them for a statistically meaningful study. The angular distributions obtained confirmed that the spin- $\frac{1}{2}$  quarks are indeed accompanied by a spin-1 partner — the gluon.

So QCD with its spin-1 gluon has indeed been proven to behave like QED with the spin-1 photon, and from this much of the stimulus to GUTs has developed. But there remained one crucial untested feature, novel to QCD and essential to the GUTs programme, and it

is this which has now been established by LEP. Whereas the electrical charge in QED is written as a number, the threefold charges ('colours') of QCD are written as  $3 \times 3$  traceless unitary matrices. (Mathematically, QED is a  $U(1)$  and QCD an  $SU(3)$  gauge theory.) This has an immediate and unavoidable consequence: the gluons of QCD can mutually interact at a point, unlike the photons of QED.

It is proof of this gluon interaction that has been lacking until now. We have long known that quarks carry a threefold charge but these could have been independent — three independent

numbers equivalent to three independent QEDs if you like — but the  $SU(3)$  matrix structure and the consequent gluon interaction are what underlie the 'asymptotic freedom' of QCD; in turn it is this that causes the strong force to become enfeebled at high energies, offers the possibility that all forces have one strength at extreme energies, and helps to forge the links between

particle physics and cosmology. Therefore, proving the existence of this gluon-interaction — the 'three-gluon vertex' — has far-reaching implications.

The clearest way that the 'three-gluon vertex' is predicted to be manifested is in the characteristics of events with 'four jets' in  $e^-e^+$  annihilations. LEP is the first electron-positron facility with enough energy and intensity to produce events containing four distinguishable jets in sufficient numbers for fruitful examination. According to QCD there are three distinct ways that 'four-jets' can arise (see figure) — (i) two gluons are radiated from a quark or antiquark, (ii) one radiated gluon converts into a quark-antiquark pair, (iii) one radiated gluon converts into a pair of gluons: this contains the 'three-gluon vertex'.

The theory of QCD predicts that each of these individual contributions has a characteristic angular distribution, momentum distribution and relative importance. Such events are rare — less than one in a hundred — but LEP has performed so well that a large sample has been accumulated. Earlier this year LEP groups reported that the data were best fitted if the three-gluon vertex contribution (iii) was present. Essentially one defined two planes — one plane defined by two of the jets, and the other plane by the other two jets. The relative orientation of these planes was only described by nonabelian theory such as QCD.

This summer the results of more extensive analysis have been reported by

\* *Hadronic Structure and Electroweak Interactions*, Amsterdam, 5–11 August 1991. *Lepton-Photon Interactions and High-Energy Physics*, Geneva, 25 July–1 August 1991.



two of these groups — the DELPHI and ALEPH collaborations — who have been able to identify the relative strengths of the three diagrams rather precisely. They find that the importance of diagram (iii) relative to the other contributions (i) and (ii) agrees best with SU(3) nonabelian theory. Here then is an independent proof that the number of colours is three.

Elegance in mathematics can be a powerful guide to progress in theoretical physics, but it is experiment that distinguishes philosophy from physical science.

## MOLECULAR GENETICS

# An ACE for hypertension

Theodore Kurtz

HYPERTENSION research finally appears to be joining the revolution in molecular medicine. On page 521 of this issue<sup>1</sup>, Lathrop and colleagues describe their use of positional mapping ('reverse' genetics) to identify two chromosome segments that contain genes regulating blood pressure in a substrain of the spontaneously hypertensive rat, the most widely used experimental model of human hypertension. In many industrialized societies, essential hypertension (high blood pressure of unknown aetiology) affects up to 25 per cent of the adult population and is a major risk factor for stroke, cardiac disease and renal failure. Most of those in the field believe that hypertension is a heterogeneous disorder, in which a variety of genetic and environmental factors interact and result in an increase in blood pressure<sup>2</sup>.

Although high blood pressure may be controlled by drugs or changes in exercise and dietary habits, it is often difficult for individuals to comply with chronic drug therapy or to make the necessary life-long changes in behaviour. In addition, strategies for the prevention of hypertension are hampered by the multifactorial nature of the disorder; for example, some individuals may be genetically susceptible to salt-induced increases in blood pressure whereas others may be susceptible to a host of different environmental determinants of hypertension. Given the enormous public health problems associated with hypertension, there is a pressing need for improved understanding of the genetic factors that predispose individuals or population groups to developing the condition.

In humans, genetic linkage studies of clinical conditions with multiple environmental and genetic components are highly problematic. In News and Views in June, Avner<sup>3</sup> described how some investigators have therefore chosen to conduct linkage studies in rodent models of complex human disorders, and he pre-

dicted that linkage methods might be used to investigate the pathogenesis of hypertension in the spontaneously hypertensive rat. Lathrop, who was part of the group which recently determined the chromosomal location of two diabetes-susceptibility genes in the mouse<sup>4</sup>, has now guided a study in the stroke-prone spontaneously hypertensive rat in which he and his colleagues<sup>1</sup> have mapped genes regulating blood pressure to chromosome 10 (*BP/SP-1*) and the X chromosome (*BP/SP-2*).

Using the same blood-pressure data set used by Lathrop's group, Lander and colleagues<sup>5</sup> have also identified markers on chromosome 10 that are linked to a gene regulating blood pressure (which they term *Bp1* and which is presumably *BP/SP-1*). In their paper, published in *Cell* last week, they additionally present preliminary evidence that a locus on chromosome 18, *Bp2*, also contributes to blood pressure. Although previous work has suggested that blood-pressure regulatory loci might also be located on rat chromosomes 13 and 20 (refs 6–9), the current studies are the first in which blood-pressure genes have been mapped using stringent statistical criteria, and the first to involve a linkage survey of most of the rat genome.

The *BP/SP-1* locus is particularly interesting in that it maps near the gene for angiotensin converting enzyme (ACE), an enzyme which mediates the synthesis of angiotensin II and the degradation of bradykinin, two vasoactive peptides involved in the regulation of blood pressure. In a large subset of patients with essential hypertension, drugs which inhibit the activity of ACE are spectacularly effective in lowering blood pressure.

Accordingly, both groups<sup>1,5</sup> propose ACE as a candidate gene contributing to the pathogenesis of hypertension, though more detailed molecular genetic experiments will be required to determine whether the ACE gene itself is the

blood-pressure regulatory locus mapped in the current linkage studies. Given the interplay of renin, angiotensinogen and ACE in the generation of angiotensin II, consideration should also be given to the possibility that blood pressure may be affected by the interaction of various alleles at these loci. With respect to the X-linked *BP/SP-2* locus, the stroke-prone spontaneously hypertensive rat appears to have fixed an allele that contributes to decreased blood pressure, at least relative to the effect on blood pressure of the allele fixed in the normotensive Wistar-Kyoto strain. This observation underscores the complexity of the genetics of hypertension and illustrates how multiple genes determine an individual's blood pressure.

Until recently, much of the research on hypertension has consisted of descriptive studies of the biochemical and physiological phenomena that accompany high blood pressure. The current reports, together with those by Rapp *et al.*<sup>6</sup> and Mullins *et al.*<sup>10</sup> on the role of the renin gene in experimental models of high blood pressure, signals the beginning of a new era in which the pathogenesis of hypertension may finally be unravelled with modern molecular genetic techniques.

But a word of caution is in order. As readers of these pages are well aware, much of the controversy that surrounds attempts to search for genes involved in psychiatric illness stems from difficulties in precisely defining phenotype. Although it may appear simple to measure blood pressure, it should be recognized that this quantitative trait can fluctuate greatly throughout the day; in linkage studies of hypertension, the optimal method for determining 'the' blood pressure of an experimental subject remains to be determined. Those who are interested in the genetics of hypertension may benefit by paying close attention to the hard lessons already learned by their colleagues studying the genetic determinants of complex disorders such as manic depression and schizophrenia<sup>11</sup>. □

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# Mantle yields its secrets

F. A. Frey

MAGNESIUM-rich glasses erupted onto the flanks of Kilauea volcano, Hawaii, as reported by Clague *et al.* on page 553 of this issue<sup>1</sup>, represent the most primitive melts recovered from Hawaiian volcanoes. They provide direct information about the composition, mineralogy and temperature of the upwelling mantle plumes that create the Hawaiian chain of volcanoes.

Sandwiched between the crust and the core, the mantle is a major part (about 68 wt%) of the Earth. To understand the formation and evolution of the Earth, it is essential to know how the present composition of the mantle varies both laterally and vertically. Indirect information comes from geophysical data, such as seismic velocities; and direct constraints arise from analyses of the rare occurrences of mantle rocks in the Earth's crust. Because most basaltic magmas in the Earth's crust formed as partial melts of the mantle, they provide another way of inferring mantle compositions. In addition, basaltic magmas are erupted in diverse geographical regions and are derived from different depths in the mantle; therefore, they can define lateral and vertical variations in mantle composition.

A primary magma is a liquid whose composition was not modified after formation by melting in a planetary interior. These are the magma compositions that can most readily be used to infer the composition and mineralogy of the inaccessible interior portions of planets. But the compositions of most primary magmas are modified during ascent by processes such as cooling and segregation of crystals, assimilation of wallrock and mixing with other magmas.

## Complexity

The compositions of these modified magmas are useful in understanding how volcanoes work, but the complexity of post-melting processes obscures information about the source material. Another complexity is that primary magmas cannot be identified without knowing or assuming the source composition. Fortunately, geophysical data, information about the mantle rocks exposed in the crust, and high-pressure melting experiments on mantle rocks and basalts allow some characteristics of terrestrial primary magmas to be inferred. Specifically, the MgO-rich mineral olivine is the dominant mineral in the upper mantle, and it is a liquidus phase of primary basaltic magmas at upper-mantle pressures. Therefore, the search for terrestrial primary magmas centres on samples that

have high MgO contents<sup>2</sup>, typically over 10 wt%. Rocks with high MgO contents could be crystallized primary magmas, but could equally be mixtures of low-MgO melt with high-MgO olivine from other sources.

Unambiguous examples of high MgO melts are quenched glasses, but few examples with MgO over 10 wt% are known; the highest MgO content<sup>3</sup> in a subaerial Hawaiian glass is about 10 wt%. Clague *et al.*<sup>1</sup> have now found volcanic glasses with up to 15 wt% MgO in sandy layers from a submarine core on the East Rift Zone of Kilauea volcano, Hawaii. Moreover, the most magnesian olivines (forsterite 90.7) in these glasses provide evidence of magmas with about 17.1 wt% MgO with an estimated eruption temperature of 1,358 °C. Such MgO-rich compositions are good candidates for primary magma compositions. Certainly, these high MgO glasses have not been extensively processed by complex, shallow magma chamber processes<sup>4,5</sup>.

Identification of the primary magma compositions that led to the development of the largest terrestrial volcanoes is especially important because the Hawaiian shields are derived from sources that are compositionally and isotopically distinct from the sources of the most voluminous terrestrial basalt, mid-ocean-ridge basalt (MORB). This is erupted at submarine spreading ridge axes such as the Mid-Atlantic Ridge and Eastern Pacific Rise.

The large volumes of Hawaiian volcanoes, combined with the compositional and isotopic differences between MORB and Hawaiian basalts, have led to the hypothesis that Hawaiian basalts are derived from mantle plumes that originate at a boundary layer beneath the mantle convection system that drives plate tectonics<sup>6-8</sup>. Therefore, Hawaiian primary magmas may provide information about the composition of the Earth's lower mantle, and may provide constraints on the role of recycled subducted oceanic lithospheres in the formation of mantle plumes<sup>9</sup>.

As a plume ascends through the convecting mantle, entrainment processes cool and dilute the plume composition. Because the MgO contents of partial melts decrease with decreasing temperature, Griffiths and Campbell<sup>10</sup> argue that it is the high MgO magmas that most accurately reflect the plume composition. Does the Hawaiian plume have a major-element composition similar to that inferred for the bulk Earth? The average of seven high-MgO glasses given

by Clague *et al.* does have an Al<sub>2</sub>O<sub>3</sub>/CaO ratio (1.24) similar to that estimated for the bulk Earth; however, the ratios of Al<sub>2</sub>O<sub>3</sub> and CaO to TiO<sub>2</sub> (5.6 and 4.5 respectively) are much lower than inferred bulk Earth values (22.6 and 17.9, respectively). Either the plume source is relatively enriched in TiO<sub>2</sub> or the partial melting process preferentially enriched TiO<sub>2</sub> in the melt. In the second case, a residual phase containing Al<sub>2</sub>O<sub>3</sub> and CaO is required (garnet?).

## Volatiles

The significant volatile content of these high-MgO glasses (0.39% H<sub>2</sub>O and 1,090 parts per million sulphur) is important. Water decreases the solidus temperature of mantle rocks, and the presence of volatiles in high-MgO glasses indicates that the magmas were not solely derived from the anhydrous residues created by melting processes such as fractional melting. However, the scatter in the MgO-FeO trend defined by the high MgO glasses (Fig. 1 of Clague *et al.*, page 554) requires either complexity in the melting process or source heterogeneity. Trace-element and isotopic analyses of these glasses are needed to resolve this ambiguity.

One of the main difficulties in understanding the evolution of the large volcanoes which form oceanic islands is that only a small fraction, about 15% at most, of the volcano is exposed above sea level. For example, the subaerial lavas of Kilauea, the most intensively studied volcano, have been examined in great detail. However, the results of Clague *et al.* show that the submarine flanks provide important new data. Nevertheless, an understanding of the origin and evolution of a Hawaiian volcano requires more than study of surficial samples. The temporal evolution can be best determined by studies of core obtained by drilling completely through the volcano<sup>11</sup>, that is, to depths of 5–10 km. □

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# Vegetable and mineral

Paul Calvert

ORGANIC polymers are soft and tough, glasses are hard and brittle. The high melting temperature of glasses has normally made them incompatible with polymers but recent work on 'sol-gel' glass preparation has allowed the synthesis of hybrids of polymers and glasses which have variously been called ormocils, ceramers and polycerams. So far most research on these materials has been of the "look what I've got" school. The burgeoning interest in these novel materials was reflected in the first symposium dedicated to them\*. Much of the debate was focused on chemists' emerging ability to control the microstructures of the hybrid materials.

The sol-gel method for silica glass is based on the hydrolysis of silicon tetroxide in an acidic alcohol solution. The first product is a soft gel containing 10-nm silica particles. As this gel is dried, it shrinks to form a porous, low-density silica matrix in which voids take up 50-99 per cent of the volume. Firing at 600 °C increases the density. Glass formed this way usually breaks up into small pieces as differential shrinkage generates high stresses during drying and densification. This is not a significant problem for thin films, and the only widespread application of sol-gel chemistry at present is for heat-reflective coatings on glass<sup>1</sup>. Work is continuing on large pieces such as mirrors and lenses. (The standard method is not restricted to silica gels but can be used to make more interesting films such as conductors, dielectrics and superconductors.)

One way of toughening the product is to incorporate flexible polymers. For example it is possible to impregnate the dried open gel with methacrylate monomer which is subsequently polymerized to form a composite of glass and polymer<sup>2</sup>. The close match between the refractive indices of the components makes this composite quite transparent. Alternatively, rubbery polymers can be impregnated with alkoxides which are then hydrolysed to form the glass component<sup>3</sup>.

Another approach is to bond the polymer covalently into the gelling silica. Wilkes and co-workers have used sol-gel reactions carried out in solutions containing dissolved polymers or oligomers which carry silane groups. These groups become incorporated into the forming silica, interlocking the two components<sup>4</sup>. And mixtures of metal alkoxides containing some polymerizable organic groups can be hydrolysed and the orga-

nic component then polymerized either thermally or photochemically<sup>5</sup>. These hybrids can be made dense and thick with no danger of cracking<sup>6</sup>.

Ellsworth and Novak<sup>7</sup> have recently described a combination of ring-opening metathesis polymerization with sol-gel formation which illustrates clearly the factors determining structure. Polymers can be made from cyclic alkene monomers combined with functional groups that can be used to tune the properties of the hybrid or can act, for example, as sensors or as optically active units. But, significantly, the authors also find that, by modifying the polymer and the conditions, it is possible to do the reaction as 'polymer-first', 'glass-first' or simultaneous processes.

The simultaneous method gives a very homogeneous material with a structure finer than is obtained using preformed polymer. It is preferable that the chemistry of polymerization and of gelation occurs distinctly, without any cross-influences. This seems to be the case when ring-opening metathesis polymerization is used but is not always

true, for instance, with free-radical polymerization or epoxy condensation.

There is a catch, which is that the products are dark brown. At the meeting, Novak mentioned, but held back from naming, a colourless alternative to the normal ruthenium catalyst.

I. A. David and G. W. Scherer (Du Pont) described making many polymer-silica blends looking for a true molecular dispersion<sup>8</sup>. Gel formation from solutions containing poly(ethyloxazoline) forms homogenous hybrids with no signs of phase separation. They looked at the reduction in brittleness of the hybrid with increasing polymer content.

As with most new materials, it is not obvious what these hybrids will be good for. The only application so far is in scratch-proof coatings for plastic lenses<sup>5</sup>. Of real potential interest are hybrids containing rhodamine laser dyes dissolved in the polymer fraction<sup>9</sup>. The solid composite replaces the stream of fluorescent dye solution that passes through the optical cavity of conventional dye lasers. The claim is that the dye lifetime is much improved. Other optical applications, including materials for electrochromic devices and for optical second-harmonic generation, are being explored.

A true materials scientist worries ab-

## The torque of Snettisham

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

SINCE 1948 a site at Snettisham in Norfolk, UK, has been yielding treasure from the last few centuries BC. Principal among the finds, discussed by I. M. Stead in the latest issue of *Antiquity* (65, 447-465; 1991), are magnificent gold torques (neck rings). The latest hoards were discovered last year, and Stead deals in detail with the curious British law of treasure trove. If an object was buried with the intention of retrieval, it is declared treasure trove, is seized by the Crown and the finder is rewarded. Otherwise, it belongs to the landowner. So had the latest hoard been part of an Iron Age treasury (in which case it would be treasure trove) or had it been left as a votive offering (in which case it would not)? A court plumped for the treasury explanation, but although Stead finds it the more satisfactory he argues that the law (which required the court to decide what was in the mind of a person burying treasure two thousand years ago) is inappropriate when applied to archaeological excavations.

Tim Lincoln

\* Meeting of the American Chemical Society, New York, 26-30 August 1991.

out thermodynamics and kinetics of phase changes, the resulting microstructures and how these affect properties. We know almost nothing about miscibility in these organic/inorganic systems except that we expect them to be always immiscible when fully reacted to glass and polymer. The method of preparing the hybrid and the course of reaction will determine when phase separation occurs, which in turn will control the scale and shape of the polymer and glass regions. The fact that the hybrids are often transparent implies that the scale of separation can be very fine, much less than one wavelength of light. Elastic-loss studies show that the glass-transition temperature of the polymer is raised as the glass content increases<sup>10</sup>. This increase may be due to inorganic material in the polymer phase or may be the result of restriction of chain motion at the surface of glass microparticles. This is of particular interest as there is much work on compatibility of polymer blends where changes in glass transition are taken to be diagnostic of mutual solubility. It takes a lot more imagination to believe in a glass dissolved in a polymer. These studies may thus force the clarification of our ideas about mixing in polymer blends. These materials are also much tougher than glasses. A glass-reinforced polymer is not a new concept, but a glass toughened by included polymer would be new and most interesting.

In the future we can expect greater control of the size and shape of the two phases; hybrids with highly elongated polymer or glass phases should be quite possible. So far most of the work has been on simple polymers combined with silica. This is the obvious combination but not necessarily the most useful. The aerospace industry is keenly interested in extending the maximum operating temperature of polymer composites. High-temperature polymers blended with glasses should give strong and tough materials with increased temperature resistance. □

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## ASTRONOMY

Elusive  $\text{H}_3^+$  ion tracked down

A. Dalgarno

THE molecular ion  $\text{H}_3^+$ , the simplest of all stable polyatomic molecular species, has been discovered in the atmosphere of Jupiter and infrared emissions from it photographed to provide images of the varying patterns of the planet's auroral activity. On pages 536 and 539 of this issue, Kim *et al.*<sup>1</sup> and Baron *et al.*<sup>2</sup>, illustrate the potential value of the  $\text{H}_3^+$  images as a probe of the deposition of energy into Jupiter's atmosphere, the connection to the plasma torus of the planet's satellite, Io, and the control exerted by the magnetic field of the planet. The emissions are seen in both the northern and southern hemispheres extending over the entire polar regions at latitudes above 60°. They have a complex morphology distinct from that of the ultraviolet emission detected during the Voyager mission<sup>3,4</sup>. There are hot spots of high intensity which do not coincide with the hot spots that have been observed in emissions from methane.

Carefully constructed theoretical models will be needed to elucidate the causes of the variations of brightness and to identify clearly the energy sources and the mechanisms that produce the radiating  $\text{H}_3^+$  molecules. A beginning has been made by Y. H. Kim, J. L. Fox and H. Porter (personal communication) who argue persuasively that the excitation arises from the transfer of vibrational energy from vibrationally excited molecular hydrogen.

This molecular ion is no astrophysical curiosity.  $\text{H}_3^+$  lies at the heart of theories of the chemistry of the interstellar gas in which it is supposed that the formation of molecules is driven by the ionization of molecular hydrogen, formed on the surface of dust grains, by cosmic rays. The cosmic rays remove one of the electrons from  $\text{H}_2$  to make an  $\text{H}_2^+$  ion which quickly reacts with  $\text{H}_2$  to form  $\text{H}_3^+$ . Mass-spectrometric studies have shown that  $\text{H}_3^+$  is the most abundant ion in a hydrogen discharge and this same reaction of  $\text{H}_2^+$  with  $\text{H}_2$  is responsible.

In interstellar gas, the formation of  $\text{H}_3^+$  is followed by proton transfers to oxygen, carbon and other heavy atoms which initiate chemical reaction sequences, leading to an array of nearly 100 interstellar molecules including such species as the hydroxyl radical OH, carbon monoxide CO, cyclopropenylidene  $\text{C}_3\text{H}_2$ , ethyl alcohol  $\text{CH}_3\text{CH}_2\text{OH}$  and the linear polyacetylenes  $\text{HC}_n\text{N}$ , all of which have been observed through their emission lines at radio and millimetre wavelengths.

Despite its central role in chemical

models,  $\text{H}_3^+$  has not yet been detected in interstellar space. Attempts have been made to find  $\text{H}_3^+$  by looking towards strong infrared stellar sources that lie near the edges of dense molecular clouds and seeking features in the spectrum that might be attributable to  $\text{H}_3^+$  absorption along the line of sight. The deuterated version  $\text{H}_2\text{D}^+$  has been found in emission at radiofrequencies but the excitation conditions are complicated and may be special so that the significance of the observation is unclear. The discovery of  $\text{H}_3^+$  in the hydrogen-rich environment of Jupiter's upper atmosphere is the most compelling empirical evidence we have that the ion must be a major constituent of dense galactic interstellar clouds.

The search for  $\text{H}_3^+$  in absorption was possible because of a combination of experimental and theoretical studies. Although not seen in emission anywhere on Earth,  $\text{H}_3^+$  has been found in absorption in the laboratory. Oka<sup>5</sup> used a frequency-difference laser system to measure the frequencies of 15 absorption lines, which he attributed to  $\text{H}_3^+$ . He could do so because Carney and Porter<sup>6</sup> had applied the variational methods of quantum mechanics to calculate, from first principles, the potential energy surface of  $\text{H}_3^+$  defining the interatomic interactions in the molecule and had solved the equations of nuclear motion to obtain the molecular energy levels to an accuracy of 50  $\text{cm}^{-1}$  (or 1 per cent), good enough to permit the identification of the experimental absorption lines. It was a remarkable achievement at the time and demonstrated the power of *ab initio* calculations. More precise calculations have since been made, with the transition frequencies now predicted<sup>7</sup> to an accuracy of better than 1  $\text{cm}^{-1}$ . The transition probabilities may be obtained from the same quantum mechanical calculations and reliable values are known for all the rotation-vibration levels<sup>8</sup>.

Thus all the basic data are available to compute both the absorption spectra and the emission spectra for specified conditions of temperature, density and radiation fields. In particular the emission spectrum corresponding to thermal equilibrium can be calculated at any chosen temperature. This ability was crucial to the discovery of  $\text{H}_3^+$  on Jupiter. Emission features due to  $\text{H}_3^+$  were seen on Jupiter in the 2-micrometre region of the spectrum by Trafton, Lester and Thomson<sup>9</sup> but not identified by them. The correct identifications and further observations were made by Drossart *et al.*<sup>10</sup>, who were able to derive a rotation-

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al temperature. The detection has been confirmed by the appearance of emission features over a broader range of infrared wavelengths at which photographic imaging may be carried out. The combination of imaging and spectroscopy offers a powerful means of diagnosing astrophysical environments.

The search for extraterrestrial  $H_3^+$  is not over. We can be confident that its observation in absorption in interstellar

clouds, at least in our Galaxy, will not be long delayed. And our comparison<sup>11</sup> of detailed calculations of thermal emission spectra leads us to a tentative identification of  $H_3^+$  beyond the Milky Way in the envelope of the supernova 1987A in the Large Magellanic Cloud. □

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## MULTIPLE SCLEROSIS

# The case of the wonky mouse

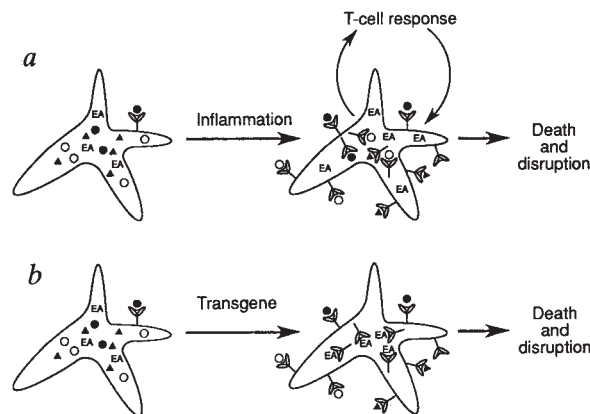
Peter Parham

MULTIPLE sclerosis and insulin-dependent diabetes are tissue-specific diseases which associate with HLA alleles and are believed to be autoimmune in nature<sup>1</sup>. It has been hypothesized that such diseases are triggered by elevated levels of HLA proteins, induced by inflammation in the target tissue<sup>2</sup>. Presentation of self-peptides to which the immune system is intolerant then stimulates a tissue-damaging T-cell response. Experiments to assess this mechanism use transgenic mice to increase expression of H-2 (the mouse homologue of HLA) in the tissues affected by disease. In earlier studies (discussed in News and Views<sup>3</sup>), targeting of H-2 molecules to pancreatic  $\beta$ -cells led to death and diabetes. But T cells were not responsible.

On page 566 of this issue, Turnley *et al.*<sup>4</sup> describe an analogous strategy to simulate multiple sclerosis in a transgenic mouse. An H-2 gene targeted to the myelin-forming oligodendrocytes of the central nervous system created 'wonky' mice which manifested severe neurological disease. Again, an autoimmune response was not evident, and Turnley and co-workers conclude that over-expression of H-2 protein directly caused the disease. But there are alternatives to this interpretation which diminish its challenge to current dogma; beyond that, the results clearly affirm the uncertainties in using transgenic mice to learn about the basis of human disease.

Multiple sclerosis is characterized by brain lesions — plaques — in which nerves are demyelinated and oligodendrocyte numbers are reduced<sup>5,6</sup>. Lymphocytes and macrophages infiltrate the plaques, and there is other evidence

for immune involvement. Susceptibility to multiple sclerosis is associated with class II HLA alleles, notably DR2, but the associations are not absolute and differ with ethnic group<sup>1</sup>. Investigation of the immune status of patients presents a confusing picture and it is uncertain whether the immune system has a formative part in the disease or responds



Schemes for the disruption of oligodendrocyte function by over-expression of a class I H-2 transgene. *a*, An autoimmune mechanism whereby increased expression of the MHC results in novel presentation patterns of tissue-specific proteins to T cells. *b*, A non-immune mechanism in which intracellular excess of H-2 molecules interferes with an essential activity (EA) of myelin formation.

secondarily to other events. Moreover, immunosuppressive therapies have met with questionable success<sup>7</sup>. In 1982 McFarlin and McFarland<sup>5,6</sup> considered that "the cause and pathogenesis of [multiple sclerosis] remains unknown" and that "No preventive measures or definitive therapies exist". The same could be said today.

Although progress towards identifying the cause of multiple sclerosis has been slow, mechanistic understanding of in-

duced demyelinating diseases of animals has rapidly advanced. Rodents immunized with myelin basic protein (MBP) contract experimental autoimmune encephalitis (EAE), which "is considered an instructive model for the demyelinating disease, multiple sclerosis in humans"<sup>8</sup>. It is also a disorder which immunologists have managed with unprecedented zeal<sup>9</sup>. CD4<sup>+</sup> T cells responding to MBP peptides presented by class II H-2 molecules cause the disease, which can be passively transferred with antigen-specific T-cell clones and pushed in almost any direction by manipulating the interaction between peptide, H-2 molecule and T-cell receptor. The mechanistic simplicity of EAE, with its compliance to molecular therapy, contrasts with the complexities of multiple sclerosis, and as yet there is little evidence to suggest that multiple sclerosis plays by the EAE rules.

Nevertheless, EAE has stimulated interest in MBP-specific T cells. These autoreactive T cells can be derived from the blood of multiple sclerosis patients and healthy individuals<sup>10</sup>, and presumably they escape the purge of negative selection because MBP peptides are not presented in the thymus. Under normal circumstances, the potential for MBP-specific T cells to attack oligodendrocytes and myelinated nerves must be kept in check. Physical separation by the 'blood-brain barrier' is probably one explanation, but may not be the whole story<sup>11</sup>. Another mechanism, suggested by the low levels of HLA expression in neural cells, is for antigen presentation to be limited in the central nervous system.

However, when they are threatened, for example, by viral infection, these tissues increase their levels of HLA through the action of interferon and other cytokines. Besides facilitating presentation of viral peptides, these events may 'inadvertently' raise presentation of MBP and other brain-specific antigens to levels that activate previously quiescent autoreactive T cells.

This mechanism (see figure) exemplifies a general hypothesis for tissue-specific autoimmunity, according to which T cells directed against tissue-specific peptides presented by aberrantly expressed HLA molecules are the root cause of disease<sup>2</sup>. Consistent with this proposal is the finding of increased HLA expression<sup>12,13</sup> in diseased tissues, including the plaques characteristic of multiple sclerosis. The evidence, however, is

### Dim view

In an unusual twist, a search for the brightest objects in the Universe, quasars, has unearthed one of the faintest — a star that is barely shining. M. Irwin, R. G. McMahon and N. Reid (*Mon. Not. R. astr. Soc.* **252**, 61p–64p; 1991) were seeking extremely red, point sources of light, which is what quasars at the limits of the visible Universe look like. But the source, BRI0021–0214, picked out by the Automatic Plate Measuring facility at Cambridge University turned out, on closer inspection, to be a star not far from us (only eight times further away than our nearest stellar neighbour,  $\alpha$  Centauri). It has eluded detection so far because it is 10,000 times fainter than the Sun. From the redness of the star, the astronomers deduce that its surface temperature is only about 2,250 K. Although not the coolest star known (that was discovered three years ago), it is one of only a handful of stars known whose mass, about a tenth that of the Sun, is only just sufficient to sustain nuclear burning. Any less, and the object is the much sought 'brown dwarf'.

### Mammoth moves

RESEARCHERS have long used the mammoth's formal name, *Mammuthus primigenius*, even though neither the generic nor the specific names have any associated type specimens. That limbo now ends with a decision of the International Commission of Zoological Nomenclature (*Bull. zool. Nomen.* **48**, 279–280; 1991). The mammoth arrived in print in 1799 as *Elephas primigenius*, but the referred material turned out to belong to another species, *E. antiquus*. The generic name *Mammuthus* arose in 1828, but type material for that, too, was lost. Hence the proposal, now approved, by W. E. Garrutt *et al.* to rehabilitate both generic and trivial names with a new type specimen — an entire skeleton of a Siberian mammoth kept at St Petersburg.

### Cold comfort

CALCULATIONS by Ph. Sindzingre *et al.* (*Phys. Rev. Lett.* **67**, 1871–1874; 1991) suggest that helium could lose its monopoly on the property of superfluidity. The problem is that other fluids freeze at temperatures far above those at which quantum-mechanical effects abolish viscosity. Molecular hydrogen has the lowest freezing point (13.8 K) after helium, and has the lowest of all molecular masses so favouring the quantum-mechanical effects. Experimenters have tried to cool droplets so quickly that they cannot freeze before superfluidity is achieved. The trick now proposed is to use clusters of just 30 hydrogen molecules or fewer to suppress crystallization. Even then the task is not easy, as the calculations indicate that temperatures as low as 1 K will be needed.

circumstantial and does not answer the fundamental question — is the immune response cause or effect?

To address this issue various groups have made transgenic mice with H-2 transgenes targeted to the cells disrupted in human disease. Following on from experiments focused on diabetes and the insulin-producing  $\beta$ -cells of the pancreas, Turnley *et al.*<sup>4</sup> have turned to multiple sclerosis and the oligodendrocyte.

A class I H-2 gene, under control of the MBP promoter, was used to make lines of transgenic mice and was expressed primarily in the oligodendrocytes. Heterozygous animals were all healthy, but the homozygotes showed distinct phenotypes. Some lines — the wonky mice — had severe neurological problems and died young, whereas others were healthy. The wonkiness was associated with lack of myelination, with reduced amounts of MBP and with a 30–40 per cent reduction in oligodendrocyte numbers. Infiltrating cells or other evidence of immune response were absent, and the animals were tolerant to the foreign transgene. Thus, a pathology similar to that of the plaques seen in multiple sclerosis was induced without accompanying autoimmunity.

Turnley *et al.* judge "the dysmyelination observed in wonky mice was primarily due to raised intracellular levels of class I MHC proteins", an interpretation (see figure) previously used to explain the non-immune diabetes in mice transgenic for H-2 expression in the  $\beta$ -cells of the pancreas. This may be the case, but wonky mice are genetically distinct from their diabetic brethren and other interpretations come to mind. Almost all mice expressing transgenic class I or II molecules in their  $\beta$ -cells became diabetic and the phenotype was inherited in a dominant fashion. Perhaps the strongest evidence that diabetes in these mice was due to intracellular overproduction of MHC protein, and not a function of integration site, was the lack of disease in mice transgenic for either class I  $\alpha$ - or  $\beta$ -chain genes but its occurrence in mice transgenic for both<sup>14</sup> ( $\alpha$ - and  $\beta$ -chains are both required to make a functional molecule). In contrast, only some of the transgenic mice of Turnley *et al.* are wonky, and the phenotype inherits recessively. This second property is difficult to reconcile with a mechanism in which overproduction of the class I protein causes severe disruption of oligodendrocyte function. If the homozygote is sick and the heterozygote healthy, this model requires that a two fold difference in an already over-expressed gene qualitatively affects an essential cellular activity.

Given the striking differences between lines of transgenic mice, the site of integration or the number of transgenes

may well have influenced the phenotype. Further insights may come from studies on two mouse strains with hereditary myelin impairment. In the *mld* strain, control of the MBP gene is perturbed — in 18-day-old *mld* mice, levels of messenger RNA are 3 per cent that of normal mice<sup>15</sup>. The defect is associated with a duplication of the MBP gene, which resulted in one normal gene and one with internal rearrangement. Although both genes have normal promoters, interference due to their juxtaposition leads to dramatically reduced levels of transcription.

Thus integration of a class I transgene carrying the MBP promoter might mimic the duplication in *mld* mice and lead to a greatly reduced expression of MBP. This could account for the reduced myelination seen in wonky mice, the recessive inheritance and the modest reduction in oligodendrocyte numbers. The disease phenotype would then be accounted for by under-expression of the MBP gene, contingent not on over-expression of the class I MHC transgene but on its site of integration. Given that the transgenic mice have multiple copies of the transgene and its MBP promoter, reduced expression of the MBP gene could also arise from competition for limiting amounts of transcription factors. The genetics of the transgenic strains is not simple — in a cross between wonky and non-wonky heterozygotes, 50 per cent of the progeny inheriting transgenes from both parents were wonky<sup>4</sup> — and may involve a combination of factors.

The second dysmyelinating strain is the *shiverer* mouse which has a homozygous deletion of the MBP gene<sup>16</sup>. Shivers start convulsing about two months after birth compared to two weeks for wonky mice. It thus might seem that fundamentally different mechanisms underlie the two phenotypes, but this may not be the case. In experiments to recreate the *shiverer* mouse using transgenic antisense MBP mRNA to knock out MBP expression, half the animals began shivering "at about 2 weeks after birth"<sup>17</sup>. So manipulation of MBP expression alone can lead to a phenotype resembling wonky. If the H-2 transgene's effect in wonky mice is directly upon endogenous MBP gene expression, rather than through its protein product, then the implications of the wonky phenotype for multiple sclerosis and autoimmunity become rather unclear.

So, to sum up, wonkiness stems from lack of myelin caused by the H-2 transgene. Its effect could be interference with the production of MBP or some other aspect of myelin formation. The transgene's role may indeed play out at the level of its protein product, as Turnley *et al.* conclude. But an alternative is for the integrated genes themselves to



disrupt the normal pattern of gene expression. The case of the wonky mouse is clearly not settled and must be adjourned to await further evidence.

In the meantime, this case confirms that although they are tractable systems for immunological tolerance, transgenic mice are hopeful monsters in autoimmunity. That transgenic mice give phenotypes resembling two human diseases without associated autoimmunity, leads Turnley *et al.* to propose that such diseases may not be caused by an immune response. The committed autoimmunologist will however counter that these transgenic mice are inappropriate mod-

els, there being many ways to skin an oligodendrocyte. Autoimmunity is commonly perceived as the secondary and unfortunate consequence of immune responses to transient, unpredictable and acute shocks inflicted by the environment. Transgenes to which mice can gradually accommodate do not readily seem to fit this bill — but as Turnley *et al.* themselves imply, common perceptions may be completely wrong. □

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## PALAEONTOLOGY

# Twilight of Hawaiian birds

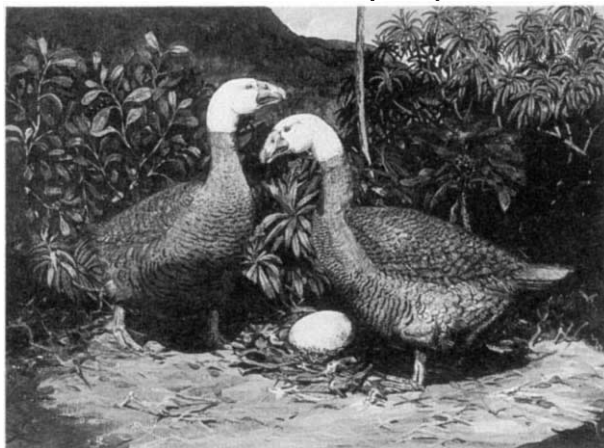
Jared M. Diamond

CONTRARY to popular belief, Pacific islands at the time of Captain Cook were not an ecologically pristine paradise, occupied by peoples living in harmony with nature until the Europeans arrived to wreak destruction. This rousseauian fantasy has been overturned during the past decade by discoveries of rich subfossil faunas that had already become extinct after the arrival of Polynesians, Melanesians or Micronesians. Now two new monographs<sup>1,2</sup> on Hawaiian bird extinctions not only tell us something of what insular evolution has achieved, but also illuminate the contentious problem of Late Quaternary extinctions on continents.

Hawaii is notorious for the extinction wave that followed the arrival of Europeans and which embraced a dozen bird species. But Olson and James's studies of late Holocene fossils now reveal an earlier wave that apparently carried off at least 50 bird species<sup>1,2</sup>; already the extinct Hawaiian bird species are outnumbering Hawaii's whole existing avifauna.

The Hawaiian discoveries include two or more flightless ibises, the astonishing total of nine flightless rails, and at least four flightless goose-like ducks with massive bills and hindlimbs. The ducks were probably herbivores ecologically equivalent

to giant tortoises or miniature horses. Preying on these creatures were six hawks and goshawk-like owls, including an eagle similar to Europe's white-tailed eagle or America's bald eagle. The endemic Hawaiian 'honey-creepers' fam-



Back to life — a painting by Julian Hume of *Ptaiochen pau*, an extinct, flightless, goose-like duck (mao-nalo) described by Olson and James. The egg in the picture is based on a fossil egg found in a lava tube.

ily, famous as an example of adaptive radiation, was represented by nearly two dozen extinct forms, most of them with finch-like or insectivore bills. (Our impression of this family as nectar-feeding specialists, based on surviving species, is evidently an artefact of differential extinction.) Most, and probably all, of these remarkable species were still alive

when the Polynesians arrived in about 300 AD, but they had gone by the time Captain Cook landed in 1778, having somehow been eliminated by the Polynesian settlement.

Until the Hawaiian discoveries were first reported in 1982 (refs 3,4), the only extensive fossil evidence for bird extinctions on Pacific islands was from New Zealand and the Chathams<sup>5</sup>. Since then, abundant fossils have been found of birds that disappeared within only a few centuries of the first human arrivals in Anuta, the Bismarcks, the Cooks, Fiji, Henderson, the Marianas, the Marquesas, New Caledonia, the Societies, the Solomons, Tikopia, Tonga and Wallis<sup>6–11</sup>. Not a single palaeontologically explored Pacific island escaped a recent mass extinction.

As for the mechanism of these extinctions, Henderson, the Chathams, and New Zealand<sup>5,6,9</sup> offer the simplest explanations. The first two were occupied by sparse populations of Polynesians who hunted, fished and gathered, but without agriculture and domestic animals, and so left forests standing. Some areas of New Zealand had dense human populations, agriculture and dogs, but much of the country was too cold and wet for agriculture or forest clearance. On all these three islands most of the extinct birds would have been targets for human hunters — colonial seabirds, for example, and large terrestrial birds, especially flightless ones such as New Zealand's moas. Like today's birds in Antarctica and the Galapagos, these vanished birds would have been easy to kill as they had never seen humans before. Only three species of small birds for all three islands combined became extinct before the arrival of Europeans; even such vulnerable species as small flightless rails survived. At least on the Chathams and New Zealand this is not an artefact of undiscovered fossil species, because almost all surviving species have already been recovered as fossils<sup>5</sup>. The mass of butchered bones of moas, seabirds and other victims at archaeological sites reinforces the evidence that most of these extinctions were a direct result of human hunting and were followed (on New Zealand and the Chathams) by extinction of large hawks dependent on the exterminated prey.

A more complex interpretation emerges for Hawaii and most other Pacific islands, which differed in supporting dense human populations who brought domestic pigs, chickens and dogs and cleared the forest for agriculture. The spectrum of extinct species is correspondingly more diverse. Birds were still

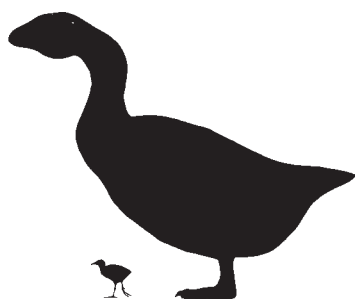
being drawn disproportionately from large flightless species, colonial seabirds, and the raptors dependent on them. But many small volant birds are included, such as the finch-like Hawaiian honey creepers discovered by James and Olson<sup>2</sup>, which could hardly have been hunted down to the last individual. Some of the small birds might have

fallen victim to avian diseases carried by the Polynesians' chickens. Ground-dwellers like the small flightless rails now being discovered as fossils on every Pacific island except those already mentioned, were probably eliminated by pigs, dogs and rats. For small arboreal species, forest destruction was the most likely factor.

These island fossils have obvious relevance for understanding Late Quaternary extinctions in North and South America and Australia, the last continents to be colonized by humans<sup>12</sup>. At the end of the Pleistocene about 11,000 years ago, North and South America lost most of their large mammals but little else, although whether this was a result of Late Pleistocene climatic changes or Late Pleistocene colonization of the Americas by humans is still debated. A similar argument has flourished for Australia and New Guinea, where human arrival around 50,000 years ago was also followed by extinction of the megafauna but of nothing much else.

Madagascar and Mediterranean islands are now also yielding fossil evidence attesting that human arrival on islands has always been accompanied by selective extinction of island megafaunas<sup>12</sup>, irrespective of whether this arrival was around 1,000 years ago (New Zealand), 1,500 (Madagascar), 3,600 (New Caledonia), 10,000 (Mediterranean islands) or 30,000 years ago (Bismarcks). Adding in the evidence for the Americas and Australia, this suggests that whenever anatomically and behaviourally modern *Homo sapiens* has reached land previously unoccupied by humans — whether a continent or an island — an extinction spasm of naive large prey has resulted.

Is it unwarranted to extrapolate from conclusions about islands to continents? Distinction between islands and continents is arbitrary: there is a continuum ranging from coral or volcanic islands with impoverished biotas, like Henderson or Hawaii, to giant continental fragments with rich biotas, like Madagascar and New Guinea, to the smallest land mass considered as a continent, Australia.



Silhouette comparison of the moa-nala *Thambetochen chaulioides* and the world's smallest known rail, *Porzana menehune*; both inhabited the island of Molokai.

lia. Could one object that biotas of islands, lacking terrestrial flightless mammals and supposedly lacking large predators, would have been especially vulnerable to human hunters? In fact, New Zealand and Hawaii had large predatory birds and Madagascar many predatory mammals, but no predator could have prepared those biotas for *Homo sapiens*.

There are objections to idiosyncratic environmental explanations for the American and Australian extinctions. First, what climatic change could have wiped out big species in every habitat from tropical rainforest to desert and savanna, instead of favouring species of some habitats and hurting species of others? Second, why did that magically unselective environmental factor, which supposedly clobbered the megafauna of all American and Australian habitats, selectively spare the megafauna of corresponding Eurasian and African habitats? Finally, why did the American megafauna that succumbed to the last Pleistocene deglaciation (the one that just happened to coincide with human arrival) resist the temptation to drop dead at each of the several dozen previous Pleistocene deglaciations?

The suggestion that island extinctions may be a good model for Late Quaternary continental megafaunal extinctions will be hotly debated by palaeontologists, but the climatic hypothesis camp must now explain why they think America's and Australia's naive biotas were uniquely resistant to the sudden onslaught of *Homo sapiens*. □

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## Motor recycling

MANY dedicated engineers are trying to perfect a clean-burning internal-combustion engine, or to create the ideal catalytic afterburner for an imperfect one. Daedalus reckons that their efforts are completely misplaced.

Modern chemical engineering reactors and electronic amplifiers, he points out, handle imperfect outputs far more intelligently. Unreacted materials or unwanted components in their output are simply fed back into the input for reprocessing. This should work splendidly in a car engine. Its emissions of particulates, carbon monoxide, and unburnt hydrocarbons are combustible, while its nitrogen oxides support combustion. So DREADCO engineers are no longer searching for a clean-burning engine. Instead, they are recycling the nasties in a dirty-burning one.

Their experimental engine has a fuel-rich injection system which gives it a black, sooty exhaust. Like charcoal, soot has an open molecular structure which adsorbs many other small molecules. It also agglomerates spontaneously into big solid particles, easily removed from a gas stream. When properly balanced, the DREADCO test-engine emits just enough soot to adsorb all the carbon monoxide, hydrocarbons, and other nasties in its exhaust. An electrostatic precipitator, or even a simple bag-filter, can then remove the whole lot in one simple step.

The resulting combustible soot is continuously resuspended in the fuel and fed back into the engine. A carburettor might balk at this thin soot-slurry, but fuel-injectors handle it with their usual aplomb. It does not burn perfectly, but then it doesn't have to: the engine needs its smoky exhaust. The engine-management system is programmed to generate enough soot under all conditions to mop up the other pollutants in the exhaust. They are recycled with the soot; only carbon dioxide and steam reach the atmosphere.

This elegant system has many advantages. The engine can be optimized for efficiency and tractability, instead of being distorted away from these engineering ideals by the need for a clean exhaust. Using all its fuel with high efficiency, it even emits less carbon dioxide and steam per unit of useful power. Poisonous combustion-modifiers, like lead anti-knock compounds, can be safely added. Indeed, they need only be added once; thereafter they will simply go round and round in the system, permanently improving the engine's operation but never escaping into the air. Engineers, environmentalists, and motor-fuel companies should all be delighted.

David Jones



## Working bloody miracles

SIR — Among the religious relics from mediaeval times that are today venerated by the Roman Catholic Church are remains of the blood of early saints. Some of these samples become liquefied from their usual clotted state on specific occasions when their containers are handled by religious leaders. A vial of the blood of Saint Januarius (San Gennaro), for example, has been liquefied every few months since 1389 in Naples. The event draws crowds of thousands and a television and media audience of millions. The phenomenon seems genuine, is well documented, and is still regarded as unexplained<sup>1</sup>.

We propose that thixotropy may furnish an explanation. Thixotropy denotes the property of certain gels to liquefy when stirred or vibrated, and to solidify



again when left to stand. Shaking or often slight mechanical disturbances thus make a thixotropic substance more fluid, even to the extent of changing it from a solid to a liquid<sup>2</sup>.

In the typical blood-liquefaction ceremony, performed at different room temperatures, the act of checking whether liquefaction has occurred comprises repeatedly inverting the glass-walled portable relic case: a shear stress is thereby applied at this critical moment. Thus a successful performance of the rite does not involve any conscious cheating. Indeed, inadvertent liquefaction events have been observed many times over the centuries during handling for repairs to the case that contains the sealed vial<sup>3</sup>.

In support of our hypothesis of thixotropy, we have been able to reproduce liquefaction of samples resembling the blood relics that we have prepared using substances available in the fourteenth century. To a solution of 25 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 100 ml of water we slowly added 10 g  $\text{CaCO}_3$ , and dialysed this solution for 4 days against distilled water from a Spectra/por tubing (parchment or

animal gut work just as well; a simple procedure<sup>4</sup> even allows us to avoid this dialysis step). The resulting solution was allowed to evaporate from a crystallization disk to a volume of 100 ml (containing about 7.5%  $\text{FeO}(\text{OH})$ ). Addition of 1.7 g NaCl yielded a dark brownish, thixotropic sol which set in about 1 hour to a gel. The gel could be easily liquefied by gentle shaking, and the liquefaction-solidification cycle was highly repeatable<sup>5</sup> (see figure).

The thixotropic property of this mixture was tested<sup>6</sup> in a CS-Bohlin rheometer (C14 coaxial cylinders system; stress sweep test, 1 Hz, 25 °C). After a 50-min setting time inside the sample cell, a shear stress (0.15–5 Pa) was applied; from the maximum in the  $G''$  (loss modulus) curve and the inflection point of the  $\delta$  (log phase) curve, we deduce a yield stress of about 4.5 Pa, corresponding to an elastic-viscous (gel-sol) transition. The same test performed after a 50-min setting time, followed by a shear stress of 5 Pa for 30 s, showed no evidence for a transition.

After making fine adjustments by adding water or NaCl, we obtained the best visual match to the contents of the Naples vial using 30 ml of this mixture in a 50-ml, round and flattened bottle. We note that ferric chloride can be found in the form of the mineral molysite on active volcanoes such as Vesuvius.

We are attempting to prepare thixotropic mixtures from other substances. Among those that have met with some success are clays (56 g finely ground bentonite<sup>7</sup> stirred in 100 ml water works well), beeswax in alcohol, and inorganic pigments in linseed or castor oil.

The chemical nature of the Naples relic can be established only by opening the vial, but a complete analysis is forbidden by the Catholic Church. Our replication of the phenomenon seems to render this sacrifice unnecessary.

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## Quantum eraser

SIR — Scully *et al.*<sup>1</sup> propose several two-source interference experiments. In one, the interference effect is wiped out by measurements that are so 'delicate' as not to disturb the system, although they yield information about the source of the detected particle. In another experiment dealing with the 'quantum eraser' concept<sup>2–4</sup>, they point out that the lost interference effect can in principle be recovered by measurements that effectively erase the information about the source of the detected particle. But, in a way, both these effects have already been observed (refs 3–5; P. G. Kwiat *et al.*, in preparation), although not described in the language used by Scully *et al.*<sup>1</sup>, and they involve the process of parametric down-conversion in a non-linear crystal, rather than the micro-maser, the principle is similar.

In the down-conversion process a beam of light of frequency  $\omega_0$  (the pump), usually from a laser, interacts with a medium having a  $\chi^2$  non-linearity in such a way that incident pump photons split into two simultaneous lower frequency photons, historically known as signal and idler, whose frequencies add up to  $\omega_0$ . The two photons usually emerge in different directions simultaneously and their state is an entangled quantum state.

The quantum eraser concept discussed by Scully *et al.*<sup>1</sup> is realized by Ou *et al.*<sup>3</sup> in a different way (Fig. 1). NL1 and NL2 are two similar nonlinear crystals optically pumped by strong, mutually coherent light beams  $V_1$  and  $V_2$ . Signal (s) and

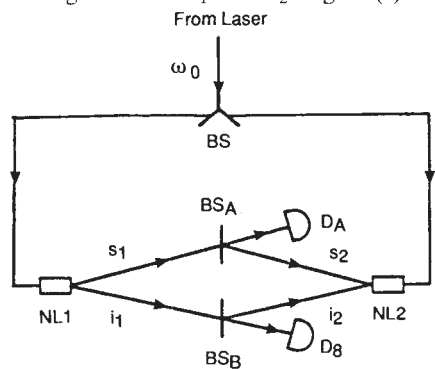


FIG. 1 Outline of the interference experiment with two down-converters<sup>3</sup>.

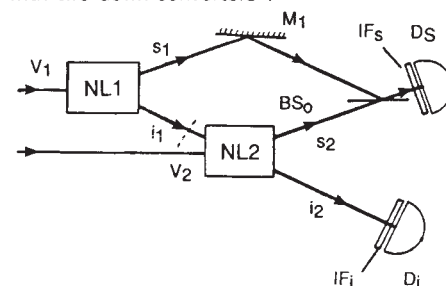


FIG. 2 Outline of the one-photon (second-order) interference experiment (from ref. 4).

idler ( $i$ ) photons emerge from both crystals. The signal photons  $s_1, s_2$  are mixed by beam splitter  $BS_A$  and the mixed beam falls on detector  $D_A$ . Similarly, idler photons  $i_1, i_2$  are mixed by beam splitter  $BS_B$  and detected by  $D_B$ . In quantum mechanics interference is the manifestation of the addition of probability amplitudes for several different, indistinguishable, possible paths. As the alternative paths from NL1 to  $BS_A$ ,  $BS_B$  and from NL2 to  $BS_A$ ,  $BS_B$  are completely indistinguishable, the corresponding probability amplitudes add. Interference should therefore show up in the joint detection probability, or the rate of coincidence detection, by  $D_A$  and  $D_B$  as a function of path difference. This was indeed observed in the experiment<sup>3</sup>.

The interference can, however, be destroyed by a 'delicate' change in the experiment, for example the removal of beam splitter  $BS_B$ , which would at first sight appear to have no effect on the signal photons. But because signal and idler photons are produced simultaneously, once  $BS_B$  is removed it becomes possible to tell from the output of  $D_B$  whether the corresponding signal photon detected by  $D_A$  comes from NL1 or NL2. Restoring  $BS_B$  and mixing the idlers restores the interference, but only in the coincidence counting rate. In the language of Scully *et al.*<sup>1</sup>, the insertion of  $BS_B$ , mixing of the idlers and subsequent detection by  $D_B$  'erases' the path information, restoring the interference.

It is possible to change the configuration of Fig. 1 in such a way that mixing the idlers restores the interference without the need for coincidence detection. An example of such an experiment is shown in Fig. 2 (refs 4,5). Here, the two nonlinear crystals NL1 and NL2 are arranged so that two idlers  $i_1$  and  $i_2$  are aligned in direction and idler  $i_1$  passes from NL1 to NL2. It is now impossible in principle to tell whether the photon detected by  $D_S$  comes from NL1 or NL2, so long as the  $i_1$  trajectory from NL1 to NL2 is not interrupted. The alignment of  $i_1, i_2$  therefore serves as the 'quantum eraser' in the experiment. However, as soon as the NL1 to NL2 coupling is broken, say by deflection of the  $i_1$  beam, or by insertion of a stop or even a sufficient time delay, it becomes possible to tell from a measurement made with an auxiliary (very efficient) detector  $D_i$  whether the photon registered by  $D_S$  comes from NL1 or NL2. Evidently, it comes from NL2 if  $D_S$  and  $D_i$  register counts simultaneously, and it comes from NL1 otherwise. The interference effect should therefore disappear when the NL1 to NL2 path is blocked. This too has been observed<sup>4,5</sup>.

In this case  $i_1$  'induces coherence' between the signals without inducing emission, even though opening or clos-

ing the path NL1 to NL2 does not physically perturb the  $s_1, s_2$  beams. Thus the state vector reflects not only what is known about the photon, but also what is knowable in principle. The 'auxiliary' measurements to identify the source or the path of the detected photon need not be carried out; it is sufficient for them to be possible, in principle, for the interference to be destroyed.

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## Actin in ribbons

SIR — We have proposed that actin filaments can be transformed by a twist and a stretch into 'ribbons', which we believe might be important for understanding the mechanism of force generation<sup>1</sup>. Our conjecture has been dismissed<sup>2</sup> on the grounds that it is inconsistent with the 'known' atomic structure of the thin filament<sup>3</sup>. This criticism is largely based on our use of the terms 'small' and 'large' to designate the axial and distal domains of the actin monomer as it packs into the ribbon. Notwithstanding the obvious confusion caused by the near identical size of the two domains, our analysis rested on the consistency of the radial position of the penultimate cysteine residue with fluorescence transfer data. Recent gold-labelling experiments<sup>4</sup> establish the similarity of actin-monomer orientation in the ribbon<sup>1</sup> and helix<sup>2</sup> states.

In agreement with DeRosier<sup>5</sup>, we take issue with the claim that the structure of F-actin has been established at high resolution as the 'atomic' model is restrained by diffraction data limited to at best 8.4 Å in the meridional direction and even less radially. Furthermore, the axial contact (the 'finger') is produced by *ad hoc* intervention without any supporting high-resolution data. We do not understand Holmes and Kabsch's insubstantiated contention<sup>2</sup> that a 30° rotation of the actin monomer would give a model consistent with F-actin but with

contacts different from those in the profilin:actin crystals, as we have not presented a high-resolution analysis. The crucial issue remains whether the genetic helix contacts are strong, as we think the balance of experimental data supports, or rather tenuous, as seen in the Holmes and Kabsch model.

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## Where next for canine virus?

SIR — Canine distemper has been recognized as a lethal infectious disease of the domestic dog for more than 200 years. Later observations have extended the known host range to include the coyote, wolf, fox, ferret, mink, skunk, raccoon, badger, panda and macaca, to name but a few. In the past 3 years, studies of mass mortalities in seals, porpoises and dolphins have revealed canine distemper or canine distemper-like infection, hitherto unknown in such marine mammals. In seals two agents are involved: canine distemper virus (CDV) and 'phocid' distemper virus, the latter perhaps a mutant of the classical dog agent.

We have investigated an episode of a fatal central nervous system disease in javalinas (collared peccaries) which live as feral animals in Arizona<sup>1</sup>. Javalinas resemble small pigs but are not directly related to pigs. We discovered that the deaths are caused by CDV encephalitis; other, healthy animals showed serological evidence of subclinical infection. By monoclonal antibody studies we found that the isolated virus is classical CDV, and not the mutant form. The javalina is also susceptible to bovine Rinderpest virus<sup>2</sup>, a morbillivirus closely related to CDV and human measles.

Clearly, CDV is a pervasive pathogen that can cause infection and disease in an unusually wide spectrum of domestic, wild and marine animals. It has recently been incriminated in some cases of Paget's disease of bone in humans<sup>3</sup>. Where will it turn up next?

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## TFIIS and strand-transfer proteins

SIR — Davies *et al.*<sup>1</sup> recently pointed out the sequence similarity between the yeast PPR2 protein<sup>2</sup>, which is required for transcriptional induction of the *URA4* gene, and transcription elongation factor SII (also called TFIIS)<sup>3</sup>. Since then, the sequence of *DST1* (ref. 4) has been published and is identical to *PPR2*. *DST1* encodes DNA-strand transfer protein- $\alpha$ , which has RecA-like activity *in vitro* in that it can promote the transfer of one strand of a double-stranded DNA molecule to a homologous single strand, and thus may be involved in recombination<sup>5,6</sup>.

It had seemed likely that *PPR2* encoded a TFIIS-related protein rather than TFIIS itself, *PPR2* being much smaller than mouse TFIIS (ref. 7). However, this conclusion was based on the original *PPR2* sequence<sup>2</sup>, which has minor differences from *DST1*. The *DST1* sequence now predicts a 309 amino-acid protein of relative molecular mass 34,800 (ref. 4), similar in size to mouse TFIIS (301 residues) and the biochemically identified yeast TFIIS protein<sup>8</sup>. Sequence similarity between the *DST1*(*PPR2*) protein and mouse TFIIS extends throughout most of its length (see figure). As with TFIIS from other species, there is a central, poorly conserved domain.

TFIIS allows RNA polymerase II *in vitro* to pass certain DNA sequences which would otherwise cause the transcription complex to pause on the template, and it is conceivable that strand transfer activity is relevant to this activity. We speculate that TFIIS discourages pausing by a direct effect on the template, perhaps by helping to denature the untranscribed DNA. Alternatively, if pausing is governed by the hybrid RNA-DNA duplex made at the pause site, the strand transfer activity might encourage elongation by promoting the displacement

of the transcript and renaturation of the template (although the transfer activity has not been shown to be active on RNA-DNA duplexes).

PPR2/*DST1* now seems an excellent candidate for yeast TFIIS, although the intriguing possibility remains that they are two distinct but related proteins. It is important to demonstrate whether purified TFIIS has strand-transfer activity, as predicted. Curiously, *DST1* overproduced in *E. coli* does not house strand-transfer activity<sup>4</sup>, possibly because of the need for accessory factors or secondary modification. The mutants *ppr2/dst1* are unable to induce transcription of the *URA4* gene<sup>2</sup> and show a meiotic (but not mitotic) recombination defect<sup>4</sup>. Clearly following the *in vitro* assays with *in vivo* studies such as these are needed to define the role of this protein in the cell.

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## Premature death

SIR — Williams *et al.*<sup>1</sup> report mass mortalities of black sea urchins (*Diadema antillarum*) in the Florida Keys and the Caribbean Sea. Their report is based on uncoordinated, qualitative or unverified observations, unlike the painstakingly assembled observations of the first reported mass mortality of this species in 1983–84 (ref. 2).

Although black sea urchins have been observed dying at several sites in the Keys and, apparently, elsewhere, Williams *et al.* make some misleading points: (1) that sea urchins are dying in the same numbers as in 1983–84; (2) that the time course for the mortality at each site is slower than for the original 1983–84 mass mortality; (3) that a potential class of pathogens has been identified; (4) that there is an implied correlation with coral bleaching; and (5) that a link is implied to an alleged mass mortality of another species of urchin in Puerto Rico. These points are not documented, and the request for additional observations

and reports can only further confuse the situation.

Williams has established an information-clearing centre to collect observations and reports of marine phenomena and disturbances over broad geographical regions. Such information must be carefully interpreted before it is reported to the community at large. Full understanding of the causes, magnitude and impact of regional events such as a mass mortality of sea urchins will come only from coordinated observation networks using standardized observation protocols.

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## Break for SCIDs

SIR — Following our report of the successful engraftment of human peripheral blood leukocytes into severe combined immunodeficient (SCID) mice<sup>1</sup>, we published a brief note<sup>2</sup> acknowledging that we had overestimated the number of human cells. We and others<sup>3–5</sup> have now studied many hu-PBL-SCID mice, and we find that human cells comprise most cells recovered from the peritoneal cavity, but a small minority (1–5%) of cells in lymphoid tissues or peripheral blood. The presence of human cells in lymph nodes and blood for at least 24 weeks after PBL injection was confirmed by PCR amplification of human genes. These data reconfirm the long-term persistence of human cells in hu-PBL-SCID mice, but in smaller numbers than we originally reported<sup>1</sup>.

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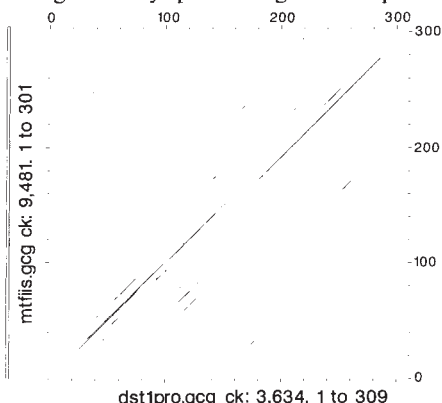
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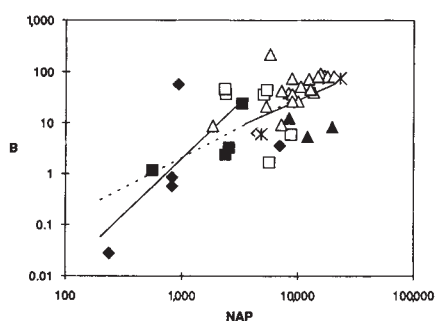


Dot-plot comparison of yeast PPR2/*DST1* (horizontal axis) and mouse TFIIS (vertical axis) amino acid sequences (window 50, stringency 19).

## Ecosystem trends

**SIR** — The relationship between primary production by plants and the biomass of herbivores has implications for the current debate on principles of population regulation. A simple, positive linear correlation is predicted by theories where herbivores are assumed to be limited by their food resources<sup>1,2</sup> and by the theory of ratio-dependent predation<sup>3</sup>. Conversely, models assuming prey-dependent predation<sup>4-6</sup> predict that the biomass of herbivores no longer increases in ecosystems productive enough to support efficient carnivores.

McNaughton *et al.*<sup>7,8</sup> concluded that herbivore biomass ( $B$ ) increases with



Relationship between net above-ground primary productivity ( $NAP$ ) and herbivore biomass ( $B$ ) using our version of the data set. Dotted line, linear regression for the entire data set ( $y = 1.19x - 3.25$ ,  $P < 0.001$ ,  $r^2 = 0.52$ ); solid lines, regressions above and below the breakpoint (see text). Filled squares, desert; filled diamonds, tundra; open triangles, tropical grassland; open squares, temperate grassland; open diamonds, old field; filled triangles, temperate forest; star, tropical forest.

increasing net above-ground primary productivity ( $NAP$ ) according to the model  $B = 10^{-4.79} NAP^{1.52}$ . We re-analysed their data set using a logarithmic regression model in which herbivore biomass levels off ( $y = a \log(x)$ , where  $y = \log B$  and  $x = \log NAP$ ). The performance of our model ( $r^2 = 0.577$ ) is practically identical to the performance of their log-linear model ( $r^2 = 0.575$ ).

We also repeated their work using the references they cited or mentioned to us in correspondence. We tried to apply the same criteria of selection, except that we also required that the systems are free from obvious, significant human impacts on trophic dynamics. When minimum and maximum herbivore biomasses were reported, we computed long-term averages if possible. If not, the reference was excluded. We accepted the Canadian high arctic<sup>9</sup> data which were rejected by McNaughton *et al.*, and we rejected several temperate data points included in their analysis. Our data set (see figure) became so different from theirs that it was impossible even to identify the

points representing the same system. Nevertheless, our general conclusions were similar. The logarithmic model with a built-in levelling off performed marginally better ( $r^2 = 0.551$ ) than the linear one ( $r^2 = 0.524$ ).

Another way to look at the situation is to test whether the slope of the regression line is significantly lower in productive systems than in barren ones. We have done this for both versions of the data set. We chose a breakpoint based on the model reported in ref. 5 and on the assumption of a shoot:root allocation ratio of 1:3 typical for tundra and grassland systems<sup>10-13</sup>, which gives a breakpoint between barren and productive systems at  $NAP = 175 \text{ g}^{-2} \text{ yr}^{-1}$  or  $3,500 \text{ kJm}^{-2} \text{ yr}^{-1}$ .

In the data set of McNaughton *et al.*, the regression line for barren systems was  $y = 1.44x - 4.60$  ( $r^2 = 0.16$ ,  $P = 0.16$ ,  $n = 14$ ) while the one for productive systems was  $y = 1.05x - 2.91$  ( $r^2 = 0.24$ ,  $P = 0.002$ ,  $n = 36$ ). In our data set, barren systems obtained the regression  $y = 2.20x - 6.30$  ( $r^2 = 0.64$ ,  $P = 0.002$ ,  $n = 12$ ), whereas the regression for productive systems was  $y = 1.01x - 2.60$  ( $r^2 = 0.16$ ,  $P = 0.03$ ,  $n = 30$ ). The two data sets thus have opposite implications about the statistical strength of the regression below and above the threshold. However, slopes are consistently lower for productive systems than for barren ones with a statistically significant difference in both cases (for the data of McNaughton *et al.*,  $t = 7.41$ ,  $P < 0.001$ , d.f. = 46; for our data,  $t = 22.95$ ,  $P < 0.001$ , d.f. = 38).

The relation of these results to various theories is complicated. The difference in the slopes is inconsistent with theories of strict resource-limitation<sup>1,2</sup> and with the theory of ratio-dependent predation<sup>3</sup>. The existence of a significantly positive slope even in productive areas is inconsistent with *laissez-faire* prey-dependent models<sup>4,5</sup> but consistent with

prey-dependent models with interference, spatial heterogeneity and/or evolutionary and behavioural responses to predation<sup>6</sup>.

Nevertheless, we warn against over-interpreting the results. The data are noisy and represent systems with various degrees of human disturbance. It is difficult to distinguish patterns from scatter and risky to assume that all patterns reflect natural dynamics.

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## No X-ray lens

**SIR** — Suehiro *et al.*<sup>1</sup> propose the use of refractive lenses for focusing X rays. Similar lenses have previously been considered<sup>2</sup>, which have not been made or used for several reasons, including the following.

(1) Because the X-ray refractive indices of all materials are very close to unity, such lenses have very long focal lengths and small numerical apertures, resulting in poor spatial resolution capabilities. The examples given by Suehiro *et al.* have numerical apertures of a few times  $10^{-5}$ , resulting in limiting resolutions of a few micrometres for 8-keV X rays. This is not a problem in the proposed use of such lenses in long synchrotron beam lines, but would be in other applications.

(2) The X-ray optical constants of materials are not well known, particularly the refractive indices. Therefore it is difficult to design and make a lens to a required specification. This is not so serious a problem for zone plates and mirrors for which the focal lengths do not depend on the refractive index.

(3) The design of refractive lenses for soft X rays, for use in, for example, X-ray microscopy, is limited by the requirement that the maximum thickness of the lens has to be less than about  $1 \mu\text{m}$  to prevent excessive absorption losses in the lens material<sup>2</sup>. This results in limits on the size and focal length of such a lens, restricting the achievable spatial resolution to a few micrometres. Zone-plate X-ray microscopes now routinely achieve resolutions of 50 nm (ref. 3), and thus it makes little sense to consider the use of refractive lenses for X-ray microscopy.

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# Deep truths and trivialities

John Ziman

**Niels Bohr's Times, in Physics, Philosophy and Polity.** By Abraham Pais. Clarendon Press, Oxford: 1991. Pp. 552. £25, \$30.

SUCH a great man of science as Niels Bohr deserves a great biography. From his quantized model of the hydrogen atom of 1913 to his interpretation of nuclear fission in 1939, he was predominant in world physics, and his Institute for Theoretical Physics in Copenhagen was the world centre of the art. He was also wise, good, fortunate and by all accounts happy. If you are looking for dirt, don't bother to knock, for there is none in his life. But if you want a scientific role model, come right in: Bohr was the real thing.

That could be the recipe for a very boring book. After all, what did happen to him? Apart from a couple of years working in Manchester at the beginning of the First World War, and escape to England and the United States in the middle of the Second World War, Bohr was firmly located in Denmark. Yes, he travelled widely, but always as a VIP on tour: Professor Pais wisely catalogues these trips concisely, except where they occasioned an important intellectual event.

Bohr was healthy and athletic. There was not even a strenuous but inspiring climb up the social ladder. His father was an eminent professor of physiology, of solid Danish ancestry; his mother came from a cultured Anglo-Jewish banking family. His wife, Margrethe, was a loving, intelligent, supportive woman. The loss before his eyes of their eldest son at the age of 17 in a yachting accident was the only deep shadow in a sunny, fruitful family life.

What would now be called managerial roles were assumed energetically and skilfully. Bohr not only built around himself and administered a superb institute: he also blazed the trail to new sources of support for science, nationally and internationally. He foresaw what was needed, and had the knack of persuading governments and foundations to put up money promptly for research posts in new fields, novel instruments such as particle accelerators, and especially havens for refugees from Nazi Germany. Of course, he was a decisive voice in the founding of CERN and its Scandinavian little brother, NORDITA.

This side of his busy, busy life is chronicled well and with justifiable admiration by Pais.

In the larger polity, however, Bohr suffered honourable defeat. He was a willing participant in the US wartime Manhattan Project to design and build the first nuclear weapons, but quickly saw the perils of a nuclear arms race. His plan was for absolute openness — as Pais puts it, *glasnost* before its time — but inconclusive dealing with Franklin

exchanges between the principal actors in this famous drama). A reader unfamiliar with the physics might find it hard going, and an expert on the history of twentieth-century physics would probably have alternative interpretations to offer on some of the details.

Nobody will dispute, however, that Bohr was the moving spirit in the collective enterprise that created our present understanding of the structure of atoms and nuclei. First, as the standard archival history shows, he produced completely new theoretical models for previously mysterious entities. Notice that these models — the electronic orbits in the atom, the nucleus as a 'liquid drop' — are drawn from classical mechanics. He was not a mathematically minded man. He did not operate creatively in abstract symbolic universes. In his later papers

there are no formulae. But his combination of physical imagery, dynamic insight and an occasional audacious postulate produced convincing explanations of apparently unconnected phenomena.

That gift sprang from deep inner sources. As a young man, he was described as "introvert, saintly, extremely friendly but shy". But there are comments on the speed with which he moved (even when pushing a bicycle), his psychic energy, and his intense compulsive engagement in one project or another. The pipe-smoking image of serenity came later, and did not deceive perceptive observers. He always insisted that he sought only to convince his co-workers, not impose his opinions. Nevertheless, younger scientists could not always cope with the immense power of his personality.

Paradoxically, it was his supreme self-confidence that made it possible for him, as he once explained to Pais, to approach every new question from a starting point of total ignorance. This inner strength also allowed him, as Albert Einstein put it, to "utter his opinions like one perpetually groping and never like one who believes he is in possession of definite truth".

But he needed to put his thoughts into words. Whatever problem he was thinking about was talked through, day after day, with a chosen colleague. These conversations — late into the night, at a railway station, on walking tours, or wherever — seem often to have been like his lectures, for Erwin Schrödinger describes Bohr "talking for minutes in a dreamlike, visionary and really quite unclear manner". But they paid off marvellously, for Bohr, for his sparring part-

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REASONS

The real thing — Niels Bohr at work at Princeton University.

Roosevelt, a famously fruitless interview with Winston Churchill, and two open letters to the United Nations in 1950 and 1956 failed to spark wider support. Whether or not such a regime ever will or could be realized, it was much too idealistic for his times.

The dimension in which Bohr lived most vividly and creatively was the world of physics. Pais tells the full story in his usual deeply informed, crisply informative and yet comfortably informal manner. He lays out the historical seedbed, reports all that is known of Bohr's ideas as they germinate and come to fruition, and summarizes later developments. This is the hard core of the book, and I find it beautifully lucid and convincingly authoritative (albeit lucid on a subject about which I know too much already — the basic quantum physics of atoms and nuclei — and authoritative on the aspect of which I am largely ignorant — the precise sequence and meaning of the

ners and for physics. Werner Heisenberg came up with his uncertainty principle after he and Bohr had "concentrated the poison" of the paradoxes of quantum theory in several months of continuous exhausting discussion. Bohr was not just a scientific impresario, drawing individual star performers to Copenhagen: he inspired and animated them intellectually in friendly, yet challenging, discourse.

The greatest of his sparring partners, his only equal in the theoretical physics of his times, was, of course, Einstein. They disagreed profoundly about quantum theory, but within the frame of an equally profound mutual respect and affection. This book is a worthy twin to Pais's splendid biography of Einstein (*Subtle is the Lord*, Clarendon Press, Oxford, 1982). Pais knew them both personally in their later years. The best passages in both books are where he describes their similarities and differences, as scientists and as people.

What is not generally appreciated is that Bohr contributed as handsomely as Einstein to our current philosophy of nature. Another brilliant passage in the book is the question-and-answer section on this contribution. Over and above his particular scientific discoveries, Bohr articulated 'the Copenhagen interpretation' of quantum phenomena, which is still, 60 years on, the best we have. It says much for Bohr's principle of complementarity — stimulated by that same heroic bout of argument with Heisenberg — that physicists and philosophers still wrestle endlessly with it.

Was Bohr, then, first and foremost a 'philosopher'? No, says Pais, and he sets out all the available evidence on this tantalizing question. Bohr's natural bent for philosophizing was manifested in and through his physics. He argued generally for 'complementarity' in other disciplines, such as psychology, biology and anthropology, as a way of talking sense about paradoxes comparable to the wave-particle duality. But he was neither a serious student of 'professional' philosophy, nor greatly influenced by it, and philosophers themselves have not taken much notice of his ideas. Indeed, as Pais notes instructively, complementarity has become so completely incorporated into our thinking as physicists that it is seldom mentioned specifically in the physics textbooks.

Nevertheless, one of the strong impressions I got from this book was of Bohr's instinctive understanding of the nature of scientific knowledge as we now conceive it. He moved on from realizing that "every description of natural processes must be based on ideas which have been introduced and defined by the classical theory" (1923) to an appreciation that the notion of a "phenomenon"

has to include both the object of study and the mode of observation. From 1927, his fundamental concern was with the language of quantum mechanics, and by 1935 he could see that "our task is not to penetrate into the essence of things, the meaning of which we don't know anyway, but rather to develop concepts which allow us to talk in a productive way about phenomena in nature". But most revealing was his aside in a family

debate: "A deep truth is a statement just as true as its opposite, in contradistinction to a triviality, the opposite of which is false: it is the task of science to reduce deep truths to trivialities." There's a man who knew what science was really about. □

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## Plotting untruths

Catherine Delano Smith

**How to Lie with Maps.** By Mark Monmonier. University of Chicago Press: 1991. Pp. 176. \$27.50, £21.95 (hbk); \$12.95, £10.25 (pbk).

Do maps tell lies? Mark Monmonier's objective is to "promote a healthy skepticism about maps". He wants to alert the map-user to what he sees as different levels of cartographic misrepresentation. His definitions are catchy: 'white lies' are those misrepresentations inherent in any attempt to portray "a useful and truthful picture" of reality; 'nefarious lies' are "deliberate falsification[s] or subtle propaganda" created by cartographers happy to manipulate the map image. Monmonier's style is fluent, even racy. His readers (especially those in the United States) will find it easy to identify with his many examples of roads and towns that exist only on paper, stylized

highway junctions and smoothed railway alignments that conceal problems of access, and suggestive shading and cunningly placed arrows intimating sources of political threat. His horror stories about what cartographers can do and have done with information on maps, and his tidbits or inside information, make a good read.

There is a dangerous simplicity in all this. Far more information can be read from a map — as from any text — than was needed in its construction. Maps are by no means unique in this respect; they are part of the problem of meaning as a whole. As the scientist Michael Polanyi pointed out some time ago, a scientific approach, as much as one in the arts, is "grounded in the decisions of its maker". But Monmonier lets key questions go unasked, particularly about the nature of cartographic truth.

For Monmonier, a good map is an 'accurate' map, one which tells the truth. The all-important matter of what truth might be, if it exists, is not broached. The book is based on the premise that there is indeed such a thing as a truthful picture and a reality that can be identified and represented accurately. The corollary is that cartographers know what is right and what is wrong. Their power lies in how they choose to present their information on a map: "you explain your needs to a freelance cartographer" and, a few days later, stare "amazed, pleased, and grateful" at his handiwork. The problem is that cartographers share the same cultural standards as society as a whole in distinguishing the valuable from the valueless. Truth is an invention. There are, for example, 'official' truths: in the United States, maps are expected to comply with 'national map accuracy standards'. To comply, a map at a scale of 1:20,000 or smaller must be checked for symbols that deviate from their correct positions by more than 1/50 inch. Ninety per cent of the points checked must meet the tolerance.

On the other hand, maps such as those showing Washington's metrorail system or London's underground network are said to have sacrificed geometrical accuracy in the interests of functional efficiency. But there are no absolute standards: quite different geometries are involved and each category of map is as



Nazi propaganda — map accompanying a story headlined 'Repatriation: Background for Peace' which appeared in 1939 in *Facts in Review*, a weekly magazine published in New York by the German Library of Information. "Germany the Peacemaker" is shown quietly reducing ethnic friction in the Baltic states by evacuating 80,000-120,000 Germans. "Germany is not afraid to correct mistakes of geography and history", the magazine observes.



accurate as the other on its own terms, the topographical map in terms of euclidean geometry, the metrorail map in terms of topographical geometry. Monmonier does not tell us this. Nor does he warn his would-be wise map-user that cartographers tend to embrace another cultural value, encoded in the idea of progress and the superiority of modern knowledge and technology.

Notions of accuracy involve value-judgements. All communication is selective and all selection subjective. It can draw from reality only as this is experienced (perceived) by the observer. In this respect, maps are no different from any written, spoken or pictorial text. For the cartographer as for the scientist, the question is what is the appropriate level of generality for the problem in hand? Does the map give sufficient information to resolve it accurately? Generalizations have to be made, but the criterion of their truthfulness has to start with their original function, that is, the purpose for which they were constructed, as well as the cultural context in which this was done. Monmonier is perfectly right that there are depths of misrepresentation on maps that may sink to the unethical, but he is wrong if he thinks they are exclu-

sive to the cartographers' paymasters. Like everybody else, cartographers imbibes society's values. They also share the responsibility to reveal and to protest about dishonest practices.

As a first step, Monmonier's book is to be greatly welcomed. While it tells us little that is new, it needs to be widely read and ingested. Even though its focus is narrow, drawing on a limited number of categories of thematic maps (for example, those used in planning, advertising and the mass media, or produced for travellers or as overt political propaganda), I hope that the book will open up the debate in ever-widening circles. Cartography needs to be integrated with mainstream critical analysis and its practitioners must learn to see its problems in relation to those dealing with the nature of knowledge, scientific observation, and the sociology of scientific paradigms. But what is needed now is a book on the meaning of maps, the nature of map knowledge and the ethics of cartographic communication. □

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## Doing it the institutional way

Frances M. Brodsky

**Cellular and Molecular Immunology.** By Abul K. Abbas, Andrew H. Lichtman and Jordan S. Pober. *W.B. Saunders*: 1991. Pp. 417, \$26.95, £19 (pbk).

**Immunology: A Short Course, Second Edition.** By Eli Benjamini and Sidney Lefkowitz. *Wiley-Liss*: 1991. Pp. 486. £17.50, \$29.95.

**Essential Immunology, Seventh Edition.** By Ivan Roitt. *Blackwell*: 1991. Pp. 368. £14.95, \$29.95.

**Immunology: A Synthesis, Second Edition.** By Edward S. Golub and Douglas R. Green. *Sinauer/W. H. Freeman*: 1991. Pp. 744. \$39.95, £29.95.

In schools of medicine, pharmacy and dentistry in the United States, it has become practically unacceptable to give lectures without providing a synopsis of their contents in a predigested form of notes known as 'the syllabus'. Undoubtedly the demand by students for a syllabus is a reaction to the enormous increase in the amount of information that they are required to absorb, but one cannot help wondering whether atrophy of their self-education skills may result from satisfying this demand.

Whatever the long-term consequence, the short-term one has been a prolifera-

tion of textbooks that began life as expanded lecture notes. Indeed, two of the immunology textbooks reviewed here are 'polished and published' syllabuses. Not surprisingly, they reflect the nature and philosophy of the institutions in which the original course was taught. For example, *Cellular and Molecular Immunology*, which evolved from the immunology course for first-year medical students in the joint Harvard Medical School/Massachusetts Institute of Technology programme, represents the ultimate set of lecture notes, detailed with Harvard thoroughness and MIT accuracy. This scholarly approach contrasts dramatically with the minimalist one of the second edition of *Immunology: A Short Course*, a product of the collaboration between the University of California at Davis and Tufts which retains the Californian attitude of 'less is more'.

These two textbooks, together with some of the other new and revised ones, provide a range of texts which are all reasonably accurate and current, but which are clearly aimed at different audiences. At the less sophisticated end of the spectrum are *Immunology: A Short Course* and the seventh edition of *Essential Immunology*. Both are directed to undergraduate or professional-school courses limited to one or two hours of lectures a week. When the first edition of *Immunology: A Short Course* was published in 1988, it seemed refreshing in its simple presentation of the complex, and sufficient for a 'bare bones'

picture of how the immune system works. But there have since been so many advances in molecular immunology that the updating of the book without the inclusion of some of these details has weakened its usefulness. Basic information noticeably absent includes proper illustrations of the folding of an immunoglobulin domain and of the HLA structure; although described in principle, these structures are only rendered as cartoons. Although a discussion of the proposed role of the  $\gamma\delta$  receptor has been added, there is no explicit indication that the role of the  $\alpha\beta$  T-cell receptor is better understood.

By contrast, although slightly eccentric in its presentation, *Essential Immunology* includes important molecular details but is still appropriate for the most basic level of teaching. Like its sixth edition, the book has more resemblance to a highly edited version of *Immunology* (Gower, 1989) by Roitt, Brostoff and Male than to its earlier editions. Although some of the illustrations follow a similarly overly complex format, most are informative and show structures where appropriate. Furthermore, Roitt provides an enthusiastic and delightful text to accompany the illustrations. He presents immunology according to a logical scheme for teaching a course with limited depth, but the format is rather unusual. He deals with B-cell and T-cell immunology in parallel, discussing the structure and genetics of antibodies, major histocompatibility complex molecules and T-cell receptors in a chapter entitled 'Molecules that recognize antigen'. Then their joint roles in cellular interactions are discussed in four chapters, each on a different aspect of 'the acquired immune response'. The book could be difficult to use as a supplement to a course that covers B-cell and T-cell immunology separately. But its organization and style should inspire those students who have to endure boring or disorganized courses.

*Cellular and Molecular Immunology* by Abbas *et al.* presents the subject at an intermediate level, being principally aimed at medical students. Thus, it is suitable as a resource book for a less sophisticated course or as a textbook for a more in-depth course. It should probably be used to accompany a dynamic set of lectures, because it is not going to turn on the uninitiated student as might the textbook by Roitt, or that by Golub and Green (discussed below), or *Immunology* by Jan Klein (for review, see *Nature* 348, 122; 1990). Yet it is the most complete and accurate textbook available for a medical-school immunology course and is surprisingly readable. Indeed, in many ways it is to immunology what Bruce Alberts *et al.*'s *Molecular Biology of the Cell* (Garland, 2nd edn,

1990) is to cell biology, providing a coherent and accurate description of the important areas and being one step removed from primary references (ideal for lecturers wishing to update their courses). The book has the conventional organization of many immunology courses, describing the antibody response, then the major histocompatibility complex, antigen presentation and T-cell recognition, followed by the T-cell response and lymphocyte ontogeny. The last section is a disease-oriented treatment of systemic immunology that does not lose sight of the molecular and cellular mechanisms.

For the PhD student, I recommend two textbooks, each having a very different approach. Most recently published is *Immunology: A Synthesis* by Golub and Green. Like Golub's previous textbooks, the book is a tribute to the history of immunology, describing the experiments that gave rise to currently accepted ideas. It is wonderful reading for those who want to know where the field has come from and the way in which immunological concepts have evolved. And for teaching purposes, the approach works well when dealing with immunochemistry, a subject that is often presented in a dull, factual fashion. But for cellular immunology it adds unnecessary complication to a field that has recently benefited from clarification at the molecular level. On the other hand, for those already familiar with the area, the discussions of the early experiments and unresolved issues, such as the nature of suppressor cells, are very useful.

The almost philosophical approach of Golub and Green is complementary to the didactic *Fundamental Immunology* by William Paul, published by Raven Press in 1989. This textbook, originating from the US National Institutes of Health, is a comprehensive collection of sophisticated review articles, less personally biased than those in *Annual Reviews of Immunology*, but pitched at the same level. Because each chapter is written by a different individual, the book lacks the coherence of Golub and Green's, providing instead thoughtful direction to the primary literature for both teachers and PhD students.

So we now have a choice of books in a variety of institutional styles, and should perhaps adapt our own syllabuses to encourage students to read these books. And if your favourite immunological style or philosophy has yet to be expressed in a textbook, you can rest assured that it will not be long until it is. □

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## Combining forces

Marian Stamp Dawkins

**The Development and Integration of Behaviour: Essays in Honour of Robert Hinde.** Edited by Patrick Bateson. Cambridge University Press: 1991. Pp. 506. £50, \$95 (hbk); £17.50, \$29.95 (pbk).

SOME scientists contribute to their field by showing up fallacious thinking and pointing out erroneous or ill-conceived assumptions. A few contribute by bringing together diverse fields of study, starting constructive new areas of research by a synthesis of different ideas. Fewer still manage to do both. Robert Hinde is one of them, and this collection of essays is a testament both to his breadth of vision and to the force of his intellect. The range of his interests is astonishing. The essays by Patrick Bateson on the processes underlying the development of behaviour, by Gabriel Horn on imprinting and by Peter Marler on the development of song in birds, all show the impact that he has had on ontogenetic studies of behaviour. Richard Andrew, in describing his own work on the effect of testosterone on behaviour, bluntly says that Hinde taught him about the causal basis of behaviour. John Fentress and J. B. Hutchison write substantial essays on hormonal and neural aspects of development, both with obvious debts to Hinde's work.

Then there is Hinde's work on social relationships. The essays on this by Thelma Rowell, Michael Simpson and Jay Rosenblatt show that here, too, his influence has been extraordinarily powerful. John Bowlby, Joan Stevenson-Hinde, Michael Rutter, Judt Dunn and Marian Radke-Yarrow all write on yet another of Hinde's interests, the development of human behaviour. Here, his ability to bring rigour and clarity to fields where there is often a danger of losing sight of these attributes is again apparent. And, as if this were not enough for one man's life, Hinde has had a considerable indirect influence on ethological fieldwork, even though he has never been directly involved in this himself. This influence is pointed out by Tim Clutton-Brock in an essay on the evolution of sex differences. By supervising the work of Jane Goodall on chimpanzees and numerous others studying primates in the wild, and insisting that data should be precise and quantitative, Hinde was responsible for lifting the quality of primate field studies so that they surpassed those on most other groups.

This is certainly not a book in which the contributors wrote what they wanted

to and then later felt they ought to 'think of something nice to say about Robert'. Quite the contrary. The whole book gives the distinct impression that all the chapters were written by people genuinely and heavily indebted to Hinde and that they chose their words carefully because each knew that Hinde himself could (and probably would) pick them up on any loose thinking. As a *fest-schrift* it is unusual, partly because the person being honoured actually contributes to the volume himself and partly because the book is so forward-looking and contains up-to-date and useful reviews on a variety of subjects. The essays by Marler on bird song and Horn on imprinting are particularly valuable in this respect. And I shall certainly recommend human sciences undergraduates to read David Hamburg's chapter on aggression and war, inspired by yet another example of Hinde's unique ability to synthesize diverse fields while being ruthlessly rigorous. In this case Hinde applies ethology to the problem of the destructive tendencies of human beings.

The whole book is woven together by what is clearly a strong thread of affection from those who have worked with Hinde. The 'memoirs' at the end by Jane Goodall and Niko Tinbergen are the clearest examples of this but it is also patently obvious in all the other chapters, even when the authors are recalling moments when Hinde was pointing out some fallacy in their work.

Pat Bateson introduces the book, and skilfully forestalls the possible criticism that behavioural studies at Cambridge University under Hinde's guidance "never used a simple explanation when a more complicated one will do". He does this by first using the quotation himself and then going on to explain its more serious implications. Hinde's emphasis on the complexity of factors that affect behaviour is neither trivial nor a retreat from testable explanations. Instead, it is a genuine reflection of the fact that the phenomena studied by ethologists are complex and deserve to be seen as such.

Quite apart from being a fitting tribute to a man who has made important contributions to so many fields, this is a well-edited and well-chosen collection of essays. Many are extremely useful and topical reviews in their own right. Most scientists would, I suspect, be more than content if in the course of their careers they had inspired half as many people to do half as many different things as Hinde has demonstrably done. □

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# Dependence of galaxy properties on viewing angle

David Burstein, Martha P. Haynes & S. M. Faber

Galaxies are three-dimensional objects projected onto the sky at random angles of inclination. Deduction of their true structure from their appearance requires an understanding of the variation of their apparent diameter, luminosity and surface brightness with viewing angle. But these variations in turn depend on galactic structure. It has taken astronomers several decades to realize that only with a direct knowledge of galactic distances can these two effects be disentangled.

SPIRAL galaxies are inherently flattened, disk-shaped, three-dimensional mixtures of stars, gas and dust. Optical pictures of spirals emphasize the light coming from stars, as modified by the extinction and reddening of dust. Galaxies are oriented at random with respect to us. Each galaxy is observed at its own viewing angle (or angle of inclination) to our line-of-sight, with some seen edge-on and some seen face-on. For every galaxy we can accurately measure two gross properties: apparent angular diameter (measured to a given surface brightness level, and defined as isophotal diameter) and apparent luminosity (within the apparent diameter). A third property, mean surface brightness, can be made by combining diameter and luminosity (to obtain luminosity per unit area) and is independent of distance.

If galaxies did not contain dust, their gross properties would project onto the sky in a manner that would be optically thin (all parts of the galaxy could be seen at all viewing angles). The optical properties of elliptical and S0 galaxies, which do not contain significant amounts of dust, behave in this manner. In contrast, the obvious existence of dust in the disks of spiral galaxies admits a wide range of possible optical-depth effects. The disks of spirals could be anything from optically thin to optically thick, depending on the radiative transfer effects when stars and dust are mixed, as well as on the viewing angle. If the disks of spirals are optically thin (case A), their isophotal diameters will increase when viewed edge-on, their measured luminosities will not change, and their surface brightnesses will show a mild increase<sup>1</sup>. On the other hand, if disks are optically thick (case B), when viewed edge-on their diameters will either be the same or slightly smaller (depending on the radiative transfer details), their luminosities will decline markedly, and their surface brightnesses will change only slightly.

These effects are large and must be known accurately before the true diameters and luminosities of galaxies can be measured. But to determine them we obviously cannot view the same galaxy from different angles. Instead, we must study statistically the 'inclination-dependent' properties of a large sample of similar galaxies observed at all viewing angles. This presupposes that a truly similar sample of galaxies can be independently selected, but, as we will show, the manner in which astronomers have selected such samples has dictated the answer they derived. This, in turn, has resulted in more than 30 years of controversy.

An understanding of the degree of optical thickness (or lack of it) of spiral galaxies is important for many problems in extragalactic astronomy. Spiral galaxies are now studied for their intrinsic properties, as well as for 'standard candles' by which the large-scale properties of the universe can be probed. If spiral galaxies are found to be optically thick, any interpretation of the stellar content of these galaxies would have to take this fact into account. If spirals are optically thick as we see them today, interpretation of spirals at large look-back times would have to model evolutionary changes not only in the stars, but also in the interstellar medium.

Modern discussion of the inclination-dependent properties

of galaxies can be traced back to Holmberg<sup>2</sup> and de Vaucouleurs<sup>3</sup>. In his classic study of spiral galaxies, in 1958, Holmberg made the explicit assumption that isophotal diameters were inclination-independent (case B), and found that these galaxies became fainter when viewed edge-on. In a separate study a year later, de Vaucouleurs made the different assumption that luminosities were constant with inclination (case A), and found that diameters of spirals increased strongly when viewed edge-on. This result was incorporated into the seminal *First Reference Catalog of Bright Galaxies* (hereafter referred to as RC1)<sup>4</sup>.

The debate begun by these two papers continued with analyses by Tully<sup>5</sup>, Heidmann *et al.*<sup>1</sup>, the *Second Reference Catalog of Bright Galaxies* (RC2)<sup>6</sup>, Burstein<sup>7</sup>, Tully and Fourqué<sup>8</sup>, Burstein and Lebofsky<sup>9</sup>, Cornell *et al.*<sup>10</sup> and Staveland-Smith and Davies<sup>11</sup>; these authors analysed samples of 30–500 galaxies. The debate was recently rekindled by the analysis of Valentijn<sup>12</sup>, which uses the largest sample to date, over 9,000 galaxies (perhaps accounting for the level of controversy that it has generated).

Here we present graphical evidence that the lack of unanimity among previous studies was due to the different ways in which the samples of galaxies used for the statistical analysis were chosen. A similar conclusion has been reached by Choloniewski<sup>13</sup> through a more mathematical treatment of the subject. The purpose of this review is threefold. First, we will show that it is not the size of the sample that determines what inclination corrections for galaxies are found, but rather the way in which galaxies are selected by apparent diameter as opposed to apparent luminosity, and it is impossible to decide without further information which approach is correct. A sample of 10,000 galaxies can reach the same incorrect answer as a sample of 100 galaxies. Second, we will use the known distances of galaxies, in a method first proposed by Burstein and Lebofsky<sup>9</sup>, to show how inclination-dependent properties of galaxies can be unambiguously determined. Third, we will briefly discuss optical-depth effects, 'opaque' galaxies and the mass-to-light ratios of the stellar populations of spirals. Part of this material has also been discussed elsewhere<sup>14</sup>.

## Galaxy luminosities and diameters

The need for inclination corrections to the optical luminosities and isophotal diameters of spiral galaxies is easily demonstrated. To do this we use a well defined subset of the sample employed by Valentijn<sup>12</sup>: 2,065 Sbc-Scd galaxies with luminosities and diameters measured by Lauberts and Valentijn<sup>15</sup> that are also contained in the original ESO catalogue assembled by Lauberts<sup>16</sup> (the ESO-LV sample). Neither diameters nor luminosities are corrected for galactic extinction, as these corrections are unnecessary for the examples used here.

Throughout this paper, we define luminosities in units of magnitudes measured in the B (blue) passband,  $B_T = -2.5 \log L_B$ . Isophotal diameters ( $D_{25}$ ) are measured, in units of tenths of an arcminute (0.1'), to a surface brightness level of

25 B magnitudes per square arcsec (this diameter corresponds to the faintest surface brightness that can be seen by the eye in the ESO sky survey photographs). The axial ratio,  $a/b$ , of the projected galaxy disk on the sky, as tabulated in the ESO catalogue, is used as an indicator of viewing angle. The ESO-LV spirals used for Figs 1, 2, 3 and 6 are subdivided into Hubble types Sbc, Sc and Sd (all disk-dominated galaxies).

Figure 1a shows  $B_T$  plotted against  $\log D_{25}$ . A strong correlation between  $B_T$  and  $D_{25}$  is seen, in the sense that apparently bigger galaxies are also brighter, but there is also a great deal of scatter. This scatter is due mainly to inclination, as we now show. Drawn on Fig. 1a are two parallel lines of slope 5 (corresponding to the distance relationship between  $B_T$  and  $D_{25}$ ). These lines are drawn to bracket the observed distribution, with the upper line closely matching the brightest limit of  $B_T$  at each  $\log D_{25}$ , and the lower line the faintest limit. Figure 1b shows the distance of a galaxy below (or above) the upper line (its luminosity residual) plotted against the logarithm of its observed axial ratio  $a/b$ . It is clear from Fig. 1b that the principal source of scatter in the plot  $B_T$  against  $D_{25}$  is the inclination of the galaxy to the line of sight. Face-on galaxies populate the upper region of the band in Fig. 1a, whereas edge-on galaxies populate the lower region.

We now know that inclination strongly affects apparent luminosity and/or diameter, but we must still ask exactly how the effect arises. Consider three possible inclination trajectories in Fig. 1a that meet at the coordinate (2.0, 12.5). If galaxies become larger with increasing inclination, but do not change luminosity (the optically thin case A), a single galaxy would move horizontally to the right with increasing inclination. If galaxies become fainter with increasing inclination but maintain constant diameter (the optically thick case B), a single galaxy would move down in luminosity, as described by the vertical line. Intermediate optical-depth effects would yield changes in both diameter and luminosity, as exemplified by the arbitrary trajectory drawn at 45°. The key question can now be stated more succinctly. When we see an edge-on galaxy in the lower band of Fig. 1a, where is the corresponding face-on galaxy in the upper band: at A, at B or at some point in between?

**Tests using distance estimates.** It is clear that Fig. 1a by itself cannot answer this question. This is where almost all previous analyses have gone wrong, by attempting to deduce inclination corrections using only luminosities and diameters themselves, with no additional information. Distance estimates to the galaxies can provide the missing clue, as can be seen from the following argument. Suppose we have identified the correct slope (that is, correct on average for the sampled galaxies) of the inclination trajectories in Fig. 1a. Then, by slicing through the band in Fig. 1a at this angle, we will have identified sets of galaxies that are all intrinsically identical—they have to be, as by construction the only different thing about them is their inclination. In particular, they must all lie at the same average distance from us, and they must all have the same intrinsic diameter if we were to view all of them face-on. Put another way, the correct set of inclination trajectories should lie parallel to the contours of identical distance and identical absolute diameter in Fig. 1a, where by absolute diameter we mean the intrinsic diameter of the galaxy.

Both these tests are powerful ways to find the correct inclination trajectories, because, in Fig. 1a, the smearing out of points along the relation is caused by two effects acting in concert. One effect is the range of distances covered by galaxies in the basic ESO catalogue. Even if all galaxies had intrinsically the same diameter and luminosity, more distant ones would look smaller and fainter, spreading the sample along the distance line (with a slope of 5 in Fig. 1a). In reality, galaxies do not all have the same diameter and luminosity; some are intrinsically small, some large, some intrinsically faint, some bright. This produces an additional smearing effect along the relation in Fig. 1a. The exact slope of this second effect cannot be learned from

the figure itself, but the lack of scatter in Fig. 1b implies that the slope is close to 5 for this sample of galaxies.

The net result is that, in Fig. 1a, we are seeing galaxies that cover a broad range of both distance and intrinsic diameter. Furthermore, and this is the crucial point, both quantities vary systematically along the distribution, with distance increasing steadily towards the lower left, and intrinsic diameter steadily towards the upper right.

Suppose now that we make an error, and we slice the band in Fig. 1a at an angle that is not parallel to the correct inclination trajectories (whichever angle that is). Galaxies from the same slice, but from the upper and lower parts of the band, will not all be at the same distance, and will not all have the same intrinsic diameter. Thus, any trend in distance and/or intrinsic diameter along a slice is a sign that the slice has been drawn at the wrong angle. This point was first realized by Burstein and Lebofsky<sup>9</sup> and reinforced by Choloniewski<sup>13</sup>.

We use these tests later to show that the true inclination trajectories in Fig. 1a are actually vertical, implying that galaxies are optically thick and that case B applies. But before doing so, it is useful to show explicitly how choosing galaxy samples in different ways can lead to very different inclination corrections,

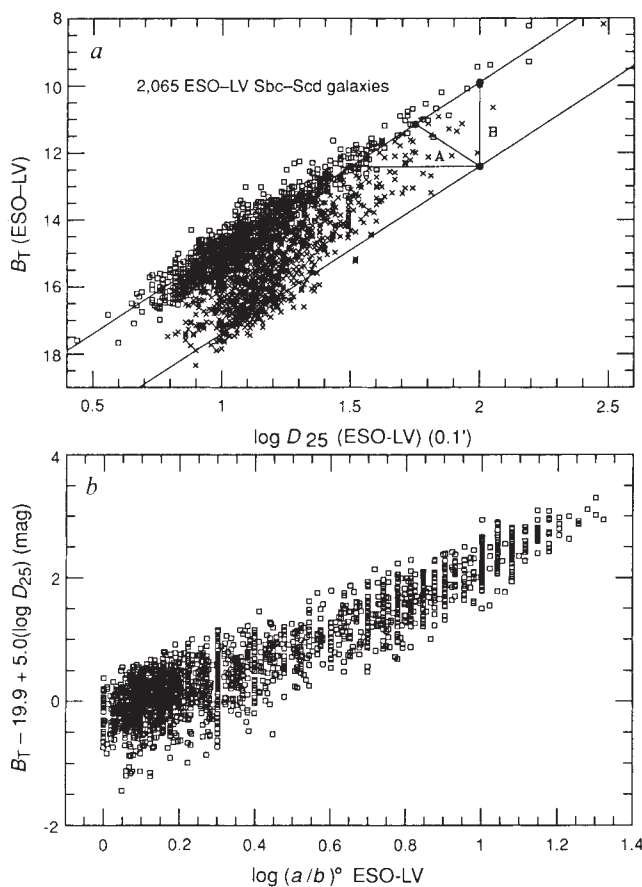


FIG. 1 a, Apparent magnitude,  $B_T = -2.5 \log L_B$ , plotted against apparent diameter at magnitude  $B = 25 \text{ arcsec}^{-2}$  (in units of  $0.1'$ ), both measured by Lauberts and Valentijn<sup>15</sup> for 2,065 Sbc-Scd galaxies in the original ESO catalogue. Two lines of slope 5 bracket the observed distribution. The three trajectories that join these two lines at 2.0, 12.5 represent three possible inclination trajectories, as discussed in the text. The trajectory labelled 'A' represents the optically thin case A, and the trajectory labelled 'B' represents the optically thick case B. b, Vertical residuals from the upper line in a, plotted against the logarithm of axial ratio  $(a/b)^0$  tabulated by Lauberts and Valentijn<sup>15</sup>. It is clear that the scatter in Fig. 1a is closely correlated with axial ratio. The data for galaxies of Hubble type Sbc are plotted as open circles, those for Sc as open squares and those for Scd as crosses.



if the data in Fig. 1a alone are used. This exercise shows concretely why apparent luminosities and diameters, analysed in isolation, cannot yield a unique solution.

**Choice of sample.** Astronomers are well aware of the dangers of selection effects and therefore prefer to analyse samples of galaxies with well understood selection criteria. To study inclination corrections for galaxies, the conventional choice has been to take all galaxies brighter than a certain apparent luminosity (luminosity-selected) or all galaxies larger than a certain apparent angular diameter (diameter-selected). We model the effect of these two strategies by picking subsamples in these two ways from the ESO-LV galaxy sample in Fig. 1a.

Figure 2 shows the results for a luminosity-selected sample that consists of the 1,310 Sbc-Scd galaxies in Fig. 1a with  $B_T$  brighter than 15.50. Panels *a* and *b* show, respectively, the apparent luminosity (defined as in Fig. 1) and apparent diameter as a function of axial ratio. For this sample, apparent luminosity evidently does not depend on axial ratio, whereas diameter increases strongly with axial ratio. Figure 3a and *b* are the analogous plots for the diameter-selected sample: the 1,555 Sbc-Scd galaxies in Fig. 1a with apparent diameters larger than one arcminute. In this case exactly the opposite trends are found: diameter is independent of axial ratio, whereas magnitude becomes fainter with axial ratio.

Figures 2 and 3 demonstrate visually what was stated earlier: the way in which one selects galaxies for study determines the kind of inclination correction derived. With a luminosity-selected sample one will find that isophotal diameter increases with inclination; with a diameter-selected sample one will find that luminosity decreases with inclination. The reason can readily be seen by taking imaginary cuts through Fig. 1a. Horizontal cuts (luminosity selection) force highly inclined galaxies to be larger; vertical cuts (diameter selection) force them to be dimmer. In the absence of distance information, no decision can be made about how the luminosities and diameters of galaxies change with inclination, despite the fact that a significant correction is demonstrably required (see also ref. 13).

### Distance-based tests for inclination corrections

We can now return to the distance-based methods defined earlier. In practice, these methods require the use of a base sample galaxies whose completeness characteristics are well known<sup>9,13</sup>. Unless one ensures that both edge-on and face-on galaxies are chosen by the same criteria, distance-related selection effects can couple with viewing-angle selection effects to render invalid even a distance-related test.

In this section we will use both distance tests discussed in the previous section. We draw slices at an angle through the observed relation between luminosity and diameter. Within each slice, we calculate the median distances and absolute diameters for galaxies as a function of inclination. (In the case of absolute diameter, this test is an extension of the classic distance-related bias discussed by Malmquist<sup>17</sup>). In principle, we ought to vary the slice angle to find the orientation that gives zero trend in both quantities: distance and absolute diameter. The correct slice angle corresponds to the true inclination trajectory. In practice, we find that vertical slices yield the zero trend to the accuracy with which it can be measured, as will be graphically demonstrated.

As we are taking vertical cuts in Fig. 1a, we need a sample that is diameter-limited, with distance estimates available for most of the galaxies. The latter requirement rules out the ESO-LV sample we have been using until now, as very few distances for these galaxies are known. Fortunately, such a sample is presented by the *Uppsala General Catalogue of Galaxies*<sup>18</sup>, which is demonstrably diameter-limited to one arcminute<sup>19</sup> and for which distance estimates in the form of radial velocities (redshifts) are available for a substantial fraction of the whole catalogue of nearly 13,000 galaxies. The UGC also has wide

sky coverage and relatively large depth in distance, both assets for these distance-related tests.

The UGC data compilation used in this analysis is part of the private data base maintained by M.P.H. and R. Giovanelli. The radial velocities (redshifts) include those contained in a tape compilation of the ZCAT kindly provided by J. Huchra in 1988, a tape version of the *General Catalog of H I Observations of Galaxies*<sup>20</sup> kindly provided by O.-G. Richter, selected other sources taken from the literature, and the private data collection of R. Giovanelli, M.P.H. and collaborators. Completeness of the redshift catalogue for UGC spirals is affected by sky coverage, in the sense that completeness approaches 90% for late-type spirals visible with the Arecibo 305-m radio telescope. Redshifts are available for 77% of UGC spirals with morphological classes Sa-Sc and 80% for Sc alone. For Scs in the declination range visible from Arecibo, redshifts are available for 91%. Where both optical and radio redshifts are available, preference is given to the latter, as the redshifts derived from radio spectra are typically more accurate than those derived by optical techniques. As most of the newer redshifts are derived from 21-cm neutral hydrogen observations, the detectability of distant objects is limited by the lower sensitivity of other radio telescopes compared with Arecibo, by the finite bandwidth of the instrumental radio response and by the frequent presence of terrestrial interference, especially at frequencies below 1,360 MHz. Most of the objects not yet detected probably lie at redshifts beyond 12,000 km s<sup>-1</sup> for declinations  $-2^\circ \leq \delta \leq +37^\circ$  and 7,000 km s<sup>-1</sup> for objects further to the north.

For our tests, UGC galaxies within each Hubble type are divided into vertical apparent diameter slices given by  $D_{UGC} \geq 3.0', 3.0'-2.0', 2.0'-1.5', 1.5'-1.0'$  and further divided within each slice into four axial ratio bins given by  $b/a = 0.00-0.25, 0.25-0.50, 0.50-0.75$  and  $0.75-1.00$ . Distances are calculated assuming a smooth Hubble flow relationship between distance and radial velocity,  $V_R$ . This assumption is sufficient for the present test, as the effects sought are much larger than the known 5-20%

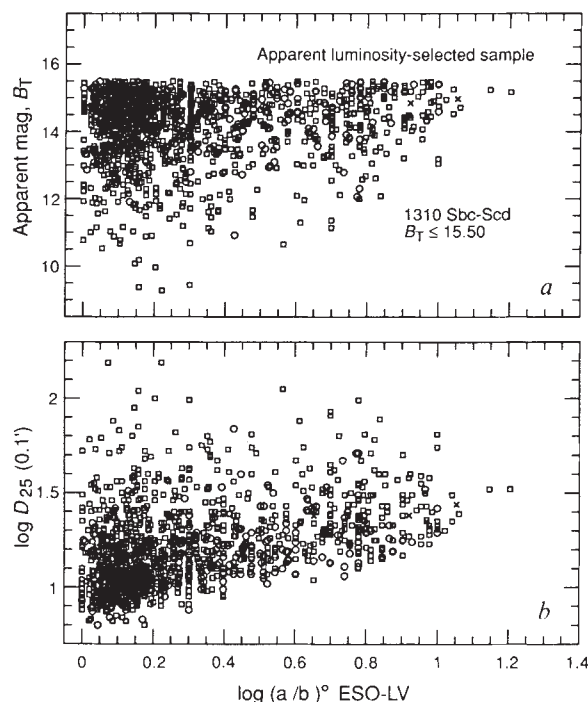


FIG. 2 Inclination dependence of *a*, *B* luminosity and *b*, isophotal diameter for luminosity-limited sample of 1,310 Sbc-Scd galaxies selected from Fig. 1a to have  $B_T \leq 15.50$ . Spirals chosen in this manner show no change in luminosity with increasing inclination, but isophotal diameter increases. Symbols are the same as in Fig. 1.

perturbations on Hubble flow distances for most UGC galaxies due to peculiar motions<sup>21</sup>. Radial velocities corrected to the rest frame of the local group are used; no differences are seen if the cosmic microwave background is used as the rest frame. Absolute diameters are calculated by taking the product of distance and diameter, and are normalized to an arbitrary Hubble constant.

The above binning procedure produces, for each Hubble type, a  $4 \times 4$  matrix defined by apparent diameter slices and axial ratio bins. Within each matrix element, median values are calculated for axial ratio, radial velocity and absolute diameter. The median absolute diameters are then normalized to unity for each Hubble type separately within each vertical diameter slice (that is, they are integrated over the 4 axial ratio bins). This permits us to compare all galaxies uniformly. The normalization constants contain information about the relative absolute diameters of galaxies as a function of Hubble type and distance, a result that is not relevant to the present discussion.

Figure 4a, b and c plots, for separate Hubble types, the values of median radial velocity (equivalent to distance) against UGC axial ratio for the galaxies within each vertical slice and axial ratio bin. The slices in apparent diameter are displaced vertically in distance because the galaxies were picked by apparent diameter; smaller-looking galaxies are therefore further away on average, as expected. Within each diameter slice, however, the median distance is independent of axial ratio. The result is the one we are seeking, and implies that the correct inclination trajectories in Fig. 1a must be vertical; that is, galaxies are optically thick (case B). Strictly speaking, we have proved this for UGC spiral galaxies, not the ESO-LV sample in Fig. 1a. The two samples are fully compatible, however, and we expect the ESO-LV sample would show the same result if distances were also available for it.

Figure 5a-f addresses the second distance test and plots the median value of normalized absolute diameter for these same Hubble types. The graphs in Fig. 5a-c are the direct analogues of Fig. 4a-c and are plotted with the same symbols denoting

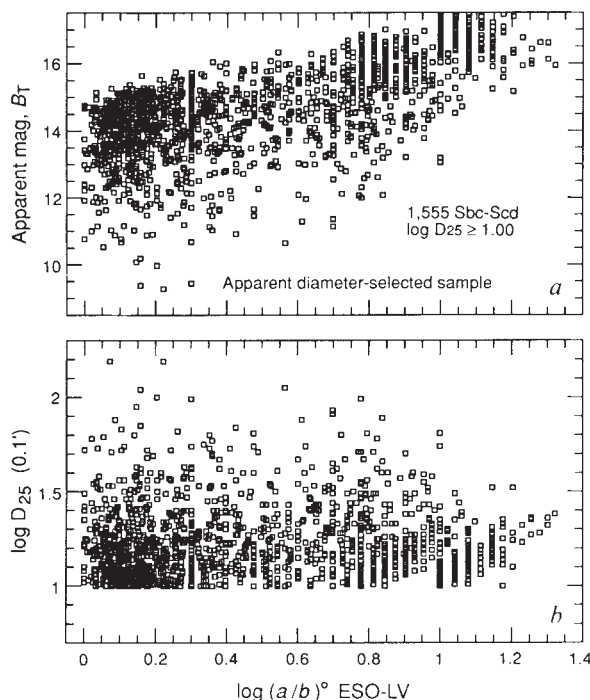


FIG. 3 Inclination dependence of *a*, *B* luminosity and *b*, isophotal diameter for a diameter-limited sample of 1,555 Sbc-Scd galaxies selected from Fig. 1a to have  $D_{25} \geq 1.0'$ . Spirals chosen in this manner show no change in diameter with increasing inclination, but luminosity decreases. Symbols are the same as in Fig. 1.

apparent diameter slices as in Fig. 4. In Fig. 5d-f we have combined all diameter slices together to improve the statistics, and calculated the medians in four different ways (the analogous figure in ref. 14 is mislabelled). The fraction of galaxies with measured distances in each axial ratio bin, for each Hubble type, is summarized in Fig. 5d-f. The sample is 70–80% complete for each galaxy type. As in the previous test, the trend in absolute diameter against axial ratio appears flat. The scatter about all of the horizontal curves in Figs 4 and 5 is a measure of the errors. Clearly, the average of all of these curves is exceedingly flat.

The tests in both Figs 4 and 5 yield a consistent result for all Hubble types and all diameter ranges: the disks of spirals do not increase their isophotal diameters when they are viewed edge-on. The consistent result for all four diameter slices is important because, first, the sample is nearly 100% complete for the three largest diameter slices and, second, each diameter slice is a separate distance-related test of a diameter-selected sample.

Could this result be an artefact of observational biases or systematic effects? It is unlikely that Nilson's UGC sample would have been biased to find smaller edge-on galaxies than face-on

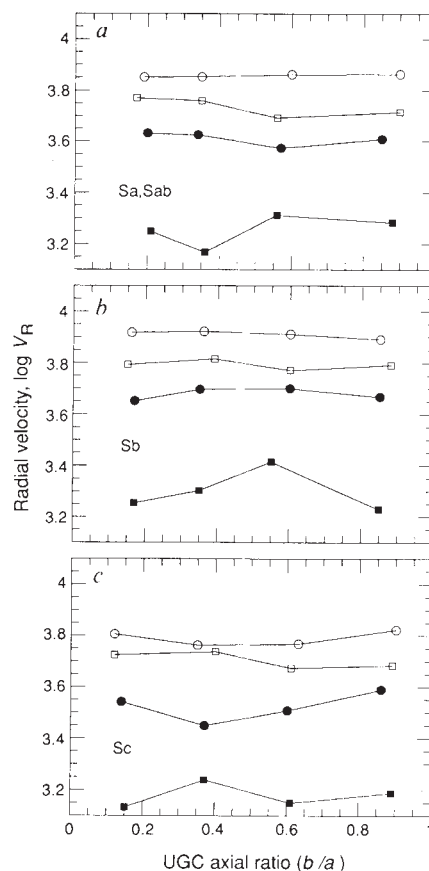


FIG. 4 Distance test using only radial velocity, for the inclination dependence of UGC isophotal diameter, for (a) Sa-Sab, (b) Sb and (c) Sc galaxies. Within each Hubble type, galaxies are divided into a  $4 \times 4$  matrix of apparent diameter and axial ratio, as discussed in the text. Median values of radial velocity (expressed as  $\log V_R$ ) are plotted against median values of UGC axial ratio, here expressed as  $(b/a)$ . Median distances ( $V_R$ ) for galaxies in the same diameter slices, but different axial ratio bins, are connected by a line: closed squares have  $D_{UGC} \geq 3.0'$ ; closed circles  $3.0' > D_{UGC} \geq 2.9'$ ; open squares  $2.0' > D_{UGC} \geq 1.5'$  and open circles  $1.5' > D_{UGC} \geq 1.0'$ . Little or no change in distance with axial ratio is seen for any diameter range within any Hubble type, showing that the correct inclination trajectories must run parallel to the diameter slices; that is, the vertical case B in Fig. 1a applies.



galaxies. Both the 'Holmberg<sup>22</sup> effect' of visually measuring edge-on galaxies to fainter isophote levels than face-on galaxies, and possible discrimination against galaxies of low surface brightness, would work to increase the diameters of edge-on galaxies relative to face-on galaxies. Similarly, incomplete redshift coverage for the smaller galaxies is balanced by the fact that the result is consistent for galaxies in all diameter slices.

In each panel of Fig. 5, two models for change of diameter with inclination are drawn: the empirically derived RC2 model<sup>6</sup>,  $\delta \log D_{\text{UGC}} = 0.235 \log(a/b)$  (note use of  $a/b$  here), and a 'straw' model  $\delta \log D_{\text{UGC}} = 0.05 \log(a/b)$ . This straw model is a reasonable limit to the accuracy to which this test can be conducted, given possible inhomogeneity in Nilson's Hubble type classification<sup>18</sup> between edge-on galaxies and face-on galaxies.

As the UGC diameter is measured near the isophote of magnitude  $B = 25 \text{ arcsec}^{-2}$  (see, for example, RC2<sup>6</sup>; Cornell *et al.*<sup>10</sup>), our result holds at an isophotal diameter similar to that used by Valentijn<sup>12</sup>. Thus, Valentijn's conclusion that diameters of spirals do not increase with inclination turns out to be correct. Valentijn, and others<sup>2,5,7</sup>, reached this conclusion without distance information. Unfortunately, it is as much a problem for science to get the right answer for the wrong reason as the wrong answer for the right reason. Like these other studies, Valentijn was correct simply because he selected his sample on the parameter that is, as it turns out, inclination-invariant, namely isophotal diameter. In absence of distance information he could not prove he was correct. As we have shown, if he had selected a sample based on apparent luminosity, he would have arrived at a very different conclusion.

The inclination-independence of isophotal diameter was also found by Burstein and Lebofsky<sup>9</sup>, Choloniewski<sup>13</sup> and de Vaucouleurs *et al.*<sup>23</sup>, the latter in their analyses of the data assembled for the *Third Reference Catalog of Bright Galaxies* (RC3; this analysis was done in collaboration with D.B.). The common thread that runs through all of these later analyses is the use of distance information to determine inclination dependencies of galaxy properties. The tests done with the RC3 data, as well as those done by Choloniewski<sup>13</sup>, support the lack of change of diameter with inclination and also show a substantial decrease in B luminosity with increasing inclination in spiral galaxies (compare this with discussion below).

## Surface brightness and optical depth

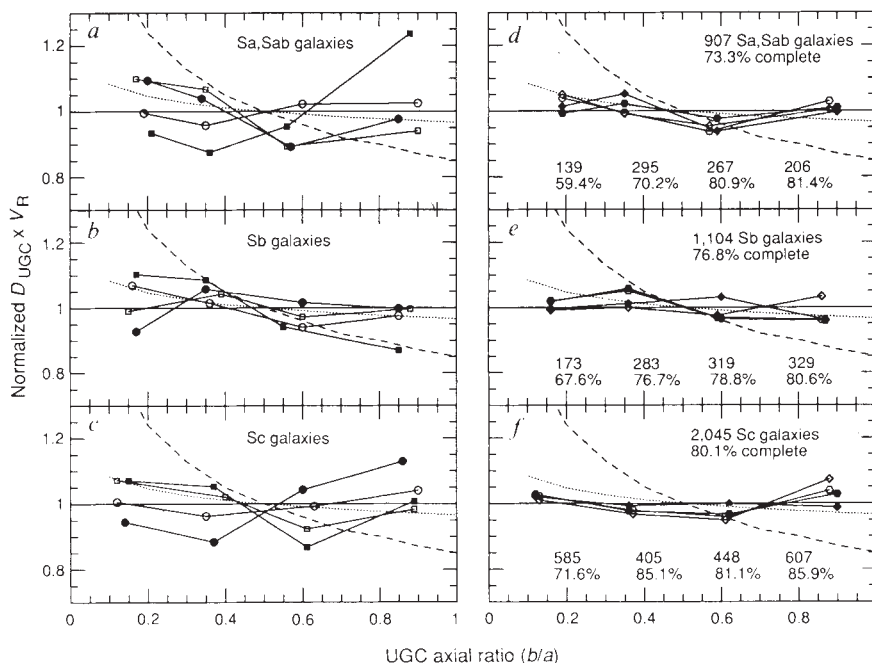
If mean surface brightness is defined as  $\Sigma_B = L_B / \pi ab$ , unpublished analysis (by D.B.) of the data used in three studies (refs 1, 2, 12) shows that  $\Sigma_B$  has little, if any, variation with axial ratio. This is shown explicitly in Fig. 6, which plots  $\Sigma_B$  against axial ratio for the 2,065 Sbc-Scd galaxies in the ESO-LV sample. No net trend is present, although there is considerable scatter. Comparable behaviour can be seen in figures used by Valentijn<sup>12</sup>.

As mean surface brightness is constant, the effect of inclination on B luminosity is simply to multiply the face-on luminosity by  $b/a$ . In other words, the B luminosities of spiral galaxies behave, on average, as though galaxies were solid disks seen at various orientations. This fact has led Valentijn<sup>12</sup> to adopt the term 'opaque' referring to galaxy disks. We prefer a term more appropriate for a radiative transfer problem, 'optically thick'. Galaxies are not opaque in the sense of being brick walls to optical radiation. We can see through parts of galaxies (for example, M31 and M33, through both of which background galaxies can be seen), just as we can easily see out through the disk of our Galaxy (ref. 24).

A galaxy that is optically thick on average need not block its light uniformly. Rather, if the picture of dust distribution in our Galaxy is typical, the distribution of dust within the disk is very patchy and irregular. The disk of a spiral can be optically thick in the same sense that its luminosity distribution can be described as exponential. Just as there are many radii along which the disk of a spiral does not look exponential, there will be many patches in the disk that are not optically thick. Moreover, the fact that galaxies behave in an optically thick manner at an isophotal diameter of 25th B magnitude  $\text{arcsec}^{-2}$  does not require that they behave in this manner at fainter, or brighter, isophotal diameters. Separate tests are needed for each successive isophotal diameter.

We would estimate that typical spiral galaxies are barely optically thick when viewed face-on, with between 1/3 and 1/2 of the optical light from a face-on spiral being absorbed by the dust, depending on competing effects between dust albedo and scattering geometry<sup>25</sup>. These effects become accentuated as a galaxy is viewed at more of an angle, such that even galaxies that are not optically thick face-on will tend to become optically thick when viewed edge-on. The number of model parameters is large enough that we must empirically determine the

FIG. 5 Alternative distance test, using the absolute diameters of galaxies, for the inclination dependence of UGC isophotal diameter. *a-c*, Median normalized absolute diameters against axial ratio for Sa-Sab (*a*), Sb (*b*) and Sc (*c*) galaxies, within the same diameter slices used in Fig. 4. Symbols are as in Figs 4*a-c*. Panels *d-f* show the median absolute diameters for all UGC galaxies of diameter 1 arcmin or larger. The four plotting symbols refer to median values calculated in four different ways: (median of  $D_{\text{UGC}}$ )  $\times$  (median of  $V_R$ ) for the whole sample ( $\bullet$ ); median of the product  $D_{\text{UGC}} \times V_R$  for the whole sample ( $\blacklozenge$ ); average of the (median of  $D_{\text{UGC}}$ )  $\times$  (median of  $V_R$ ) for all four diameter slices ( $\diamond$ ); and average of the median values of  $D_{\text{UGC}} \times V_R$  for all four diameter slices ( $\circ$ ). The dashed line drawn in all six figures represents the inclination dependence of diameter predicted in the RC2 model<sup>6</sup>,  $\delta \log D_{\text{UGC}} = 0.235 \log(a/b)$ , and the dotted line represents a 'straw' model:  $\delta \log D_{\text{UGC}} = 0.05 \log(a/b)$ . No type of spiral is observed to change diameter with inclination as a class, and the straw model is taken as a reasonable upper limit to any real effect. (The figure in Burstein<sup>14</sup> analogous to *d-f* is mislabelled.)



inclination corrections to diameters and luminosities to constrain these models, rather than vice versa (for example, as attempted in refs 1 and 26). If the dust is similar to that in our Galaxy, the models made by Bruzual *et al.* would indicate that, for face-on galaxies with optical depths near unity, blue minus visible colours ( $B - V$ ) would become  $\sim 0.1$ – $0.3$  mag redder (in the standard astronomical notation).

The resulting corrections for extinction would increase the stellar luminosity of a spiral galaxy, but the concomitant colour correction would also make the galaxy bluer. Larson and Tinsley<sup>27</sup> show that the stellar mass-to-light ratio is a strong function of colour, with bluer colours (hotter stars) having smaller mass-to-light ratios. From Table 1 of Larson and Tinsley<sup>27</sup>, a bluing in  $B - V$  of 0.2 mag for disks of spirals with observed colours  $B - V \approx 0.6$  would correspond to changes in the inferred mass-to-light ratio in the V passband by factors of 2–4, depending on the precise history of star formation. In contrast, correcting for the internal extinction at V could increase the total luminosity by as much as a factor of 2–3. Ironically, within uncertainties of a factor of 2, spiral galaxies modelled either as optically thick or optically thin yield similar amounts of stellar mass because the inclination effect and colour corrections approximately cancel for the optically thick case. Hence, optically thick spirals are not an obvious solution to the 'dark matter' problem in spiral galaxies, contrary to the suggestion of Valentijn<sup>12</sup>.

Moreover, even if typical spiral galaxies have disks that are optically thick, the present data do not require that the disk of every spiral be optically thick. As shown in Fig. 6, a wide intrinsic range of mean B-surface brightness exists at all inclinations for galaxies of similar Hubble type, with edge-on galaxies having a smaller range than face-on galaxies. As mentioned above, this

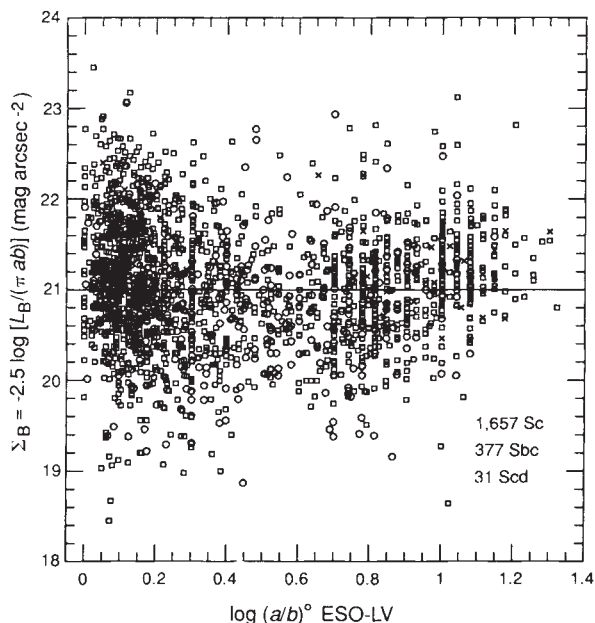


FIG. 6 Effective surface brightness,  $\Sigma_B = L_B / (\pi ab)$ , expressed in terms of B magnitude arcsec<sup>-2</sup>, plotted against  $\log(a/b)^0$  for the galaxies in Fig. 1, coded by Hubble type as in Fig. 1. The number of galaxies in each Hubble type is given. Several points should be made about these data: first, the apparent 'quantization' of axial ratio here and in Fig. 1 arises because the axial ratios used here were 'adopted for the determination of photometric parameters related to ellipses' (ref. 15, p. 19). As the values of  $(a/b)^0$  are fitted by eye, some axial ratio 'quantization' is evident. Second, the relative paucity of galaxies with  $0.4 < \log(a/b)^0 < 0.8$  is because galaxies are smoothly distributed in  $(a/b)$ . Third, it is plausible that the rise in surface brightness for galaxies with  $\log(a/b)^0 > 0.8$  is due to the inclusion of galaxies with different-sized bulges. Bulges are generally optically thin, so edge-on galaxies with large bulges have both larger apparent values of axial ratio and higher mean surface brightness than edge-on galaxies with smaller bulges.

could result if some galaxies are more optically thick than others, with all galaxies becoming optically thick when viewed edge-on. Differences in optical thickness could arise, from intrinsic differences in the kinds of dust and in the kinds of stellar populations that can exist in spiral galaxies. We have only begun to scratch the surfaces of spiral galaxies. Much more detailed work, especially infrared imaging and spectroscopy, will be needed to decode the clues provided the intrinsic range of B magnitude surface brightnesses among spirals.

From Fig. 1b, it is apparent that the luminosities of Sc galaxies become fainter by  $\sim 2.5$  magnitudes for a change in axial ratio of a factor of 10. If the slope in Fig. 1b were exactly 2.5, this would imply that the mean surface brightness was constant as a function of inclination for this sample, as confirmed by Fig. 5. Although it is now clear that spiral galaxies become much fainter when viewed edge-on, we feel it would be unwise to draw firm conclusions without performing specific distance tests for luminosities. In particular, luminosities of galaxies with larger bulges could be inclination-dependent in a different way from luminosities for the disk-dominated galaxies used in Figs 1 and 6 (bulges are relatively dust-free). Unfortunately, the UGC does not contain reliable luminosities for edge-on galaxies, and the ESO-LV sample does not have many redshifts. Accurate determination of inclination dependence of luminosity therefore awaits the assemblage of an appropriate data sample.

## Conclusions

Our paper concerns a controversy that has existed for the past 30 years and was recently rejuvenated by Valentijn<sup>12</sup>. We show that most previous attempts to derive the inclination dependence of luminosity and diameter for spiral galaxies could have been afflicted by severe sample selection effects. In the absence of distance information, analysis of a diameter-selected sample will reach one conclusion, and the same kind of analysis of a luminosity-selected sample will reach the opposite conclusion.

Knowledge of the distances of the galaxies is required to break this circle of confusion, as first pointed out in ref. 9 and confirmed in ref. 13. We show that, when distances are used, well-selected samples of spirals can yield reliable inclination corrections. Distance-related tests are applied to diameter-limited samples of the UGC, with the result that we find that apparent diameter is independent of inclination for Sa–Sc spirals. This result further implies that the luminosities of spirals are inclination-dependent, becoming much fainter as they are viewed more edge-on. How much fainter will require analysis of distances for a luminosity-selected sample of galaxies.

Radiative transfer modelling of dusty galaxies can help us to interpret the effects we are observing, but there are simply too many variables in the models for us to expect them to yield accurate predictions. Rather, we see the need to use observed trends to constrain the models. This will require better and more detailed luminosity and diameter data for more galaxies. The inclination dependence of luminosities and diameters defined at other isophotal surface brightnesses should yield valuable information about the radiative transfer properties of the dust.

Moreover, reliable inclination corrections will be available for all distance-dependent parameters for all galaxy types when larger, complete samples of galaxies with measured redshifts become available. As redshifts of galaxies are being measured rapidly for cosmological studies, it is expected that such complete samples will be available soon. We have already established that isophotal diameter of 25th B magnitude arcsec<sup>-2</sup>,  $D_{25}$ , does not change with inclination for spiral galaxies. We interpret this lack of change as signifying that spirals are moderately optically thick, as opposed to the term 'opaque' introduced by Valentijn<sup>12</sup>. Optically thick galaxies are also likely to be reddened. When effects of both extinction and reddening are corrected, the inferred (and uncertain) stellar mass should not change significantly from that which would be inferred without any extinction corrections to magnitude. □



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# Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats

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The spontaneously hypertensive rat and the stroke-prone spontaneously hypertensive rat are useful models for human hypertension. In these strains hypertension is a polygenic trait, in which both autosomal and sex-linked genes can influence blood pressure<sup>1-7</sup>. Linkage studies in crosses between the stroke-prone spontaneously hypertensive rat and the normotensive control strain Wistar-Kyoto have led to the localization of two genes, *BP/SP-1* and *BP/SP-2*, that contribute significantly to blood pressure variation in the F<sub>2</sub> population. *BP/SP-1* and *BP/SP-2* were assigned to rat chromosomes 10 and X, respectively. Comparison of the human and rat genetic maps indicates that *BP/SP-1* could reside on human chromosome 17q in a region that also contains the

angiotensin I-converting enzyme gene (*ACE*)<sup>8</sup>. This encodes a key enzyme of the renin-angiotensin system<sup>9</sup>, and is therefore a candidate gene in primary hypertension. A rat microsatellite marker of *ACE* was mapped to rat chromosome 10 within the region containing *BP/SP-1*.

PRIMARY hypertension represents a major public health issue owing to its prevalence (20-30% of the population)<sup>10</sup> and its deleterious long-term complications<sup>11</sup>. Family and twin studies have demonstrated that hypertension is inherited as a multifactorial trait and that several different genes may be involved in the regulation of blood pressure and the pathogenesis of hypertension<sup>12</sup>. Difficulties that have been encountered in attempts to identify these genes in humans include the problems of phenotype definition, ascertainment of informative families for genetic studies, and the likely heterogeneity of the disease.

Investigations of genetically hypertensive inbred rodent strains can partially overcome problems inherent in human studies. Controlled crosses show that a small number of genes are responsible for blood pressure differences between

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hypertensive and normotensive rat strains<sup>1-7,13-18</sup>. These results demonstrate that the genes can be localized and then characterized for their physiological effects. Comparative mapping allows identification of the homologous genes in man and hence of their role in human hypertension.

The relationship between blood pressure and physiological or biochemical phenotypes<sup>4,14,15</sup>, as well as DNA polymorphisms of specific candidate genes<sup>7,16-18</sup>, have been examined in several crosses. But linkage investigation in the rat has been hampered by a lack of informative markers. Although the candidate gene approach has the advantage that sequences can be directly related to a known physiological function, it is restricted to characterized genes. As most genes influencing blood pressure are still unknown, linkage with random markers will be necessary for studying the genetic component of hypertension. Once linkage is established, candidate genes for experimental and human hypertension can be identified by comparative mapping.

We use here the stroke-prone spontaneously hypertensive rat (SHRSP) to localize genes involved in SHRSP hypertension. We have developed a set of mapped minisatellite and microsatellite markers characterized in F<sub>2</sub> populations from SHRSP and Wistar-Kyoto (WKY) crosses, in which 2-4 major genes are thought to influence blood pressure<sup>2,3,7,13</sup>. Linkage studies with these markers has enabled us to localize two loci that contribute significantly to basal and NaCl-loaded blood pressure levels in the F<sub>2</sub> population.

### Phenotype characterization and marker loci

We measured physiological and biochemical parameters in a cohort of 115 F<sub>2</sub> hybrid animals, 57 originating from WKY male and SHRSP female progenitors (cross 1), and 58 from WKY female and SHRSP male progenitors (cross 2). As SHRSP animals show increases in blood pressure in response to dietary sodium and WKY show little or no response<sup>19-23</sup>, both basal blood pressures and blood pressures after twelve days of oral loading with NaCl (NaCl-loaded blood pressure) were examined (Fig. 1).

To test for linkage, we characterized 181 marker systems in the F<sub>2</sub> population. The markers were obtained from a combination of 11 minisatellite probes that detect multiple bands (fingerprint patterns) under low-stringency hybridization conditions<sup>24</sup>, 51 microsatellite markers developed and mapped for this study, and a restriction-fragment-length polymorphism (RFLP) of the renin gene<sup>7</sup> (Fig. 2). Fingerprint bands are scored as present (+) or absent (-) in F<sub>2</sub>, where the presence of the band corresponds to two possible underlying genotypes (+/+ or +/-), so the + allele is dominant. Microsatellite markers are based on short tandem repeat elements, and they are readily characterized by polymerase chain reaction (PCR). We developed such markers in the rat because they are widely distributed and highly polymorphic in the human<sup>25,26</sup> and murine genomes<sup>27</sup>.

### Autosomal linkage

For the detection of linkage, blood pressure was considered as a quantitative phenotype to which we applied both analysis of variance (ANOVA) and combined linkage and segregation analysis (Table 2). We chose  $P = 0.0001$  as a critical limit for detection of linkage to reduce type-1 error (false positive linkages).

Microsatellite markers of the growth hormone promoter region (RatGHP), and the fast nerve growth factor receptor (RatNGFR) were significantly linked ( $P < 0.0001$ ) to basal diastolic blood pressure, and to systolic and diastolic blood pressures after NaCl loading; tests for linkage to systolic basal blood pressure gave  $P = 0.0002$  with RatNGFR (Table 2). From our data, RatGHP and RatNGFR were also linked, with a maximum lod score of 19 at 12% recombination. The two markers and others in the same linkage group were assigned to rat chromosome 10 by somatic cell hybrids, which confirmed

the previous localization of RatGHP<sup>28</sup>. No other autosomal markers showed significant linkage to blood pressure variables, with the exception of RatACE, as described later.

The autosomal linkage results suggest that a locus (denoted as *BP/SP-1*) on chromosome 10 influences blood pressure in our crosses. Blood pressure measurements were compatible with a dominant effect of the *BP/SP-1* allele from SHRSP in the quantitative genetic analysis. The *BP/SP-1* locus was estimated to account for 22% of systolic and 21% of diastolic blood-pressure variances after NaCl loading, corresponding to 15 mm Hg and 13 mm Hg of difference between the genotype means in the genetic analysis (results not shown). For basal systolic and diastolic blood pressures, the figures are 12% (8 mm Hg) and 17% (9 mm Hg) respectively.

Figure 3 shows the distribution of blood pressure measurements as a function of the presence or absence of the SHRSP allele at the RatGHP locus (which gave the greatest evidence of linkage to NaCl-loaded blood pressure values).

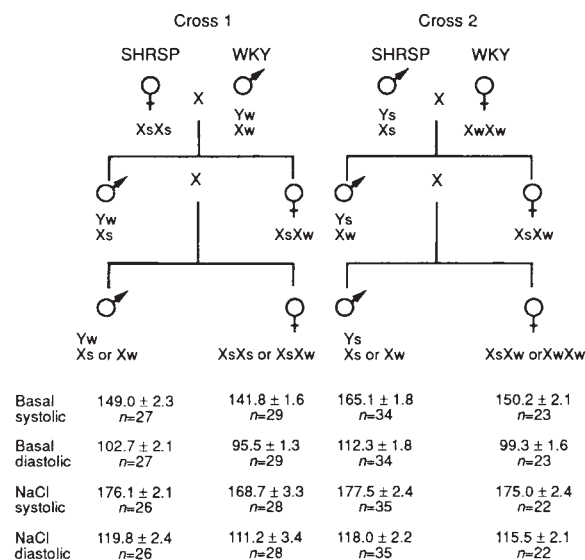


FIG. 1 Transmission of sex chromosomes, and statistics on blood pressure measurements by cross and sex. The two crosses differ in transmission of the sex chromosomes: males from cross 1 (SHRSP female × WKY male) have received the WKY Y chromosome (Y<sub>w</sub>) whereas males from cross 2 (SHRSP male × WKY female) have received the SHRSP Y chromosome (Y<sub>s</sub>). Males from both crosses receive X-chromosome alleles from either SHRSP (X<sub>s</sub>) or WKY (X<sub>w</sub>); females from cross 1 are either X<sub>s</sub>X<sub>s</sub> or X<sub>s</sub>X<sub>w</sub>, while females from cross 2 are either X<sub>s</sub>X<sub>w</sub> or X<sub>w</sub>X<sub>w</sub>. Breeding conditions to maintain the SHRSP and WKY lines, and construction of the F<sub>2</sub> have been described<sup>7</sup>. Briefly, F<sub>2</sub> animals were maintained on regular rat food containing 0.2% Na<sup>+</sup> (Altromin, Germany). F<sub>2</sub> animals underwent catheterization of the right femoral artery at 4 months of age, when the initial rapid development of hypertension has entered an asymptotic phase (unpublished result), and three sets of blood pressure measurements were taken on two consecutive days, using a model P23PD Statham pressure transducer (Gould-Statham) connected to a Grass Model 7B polygraph (Quincy, MA), calibrated before each measurement. Animals were shielded from the observer, and measurements were taken only while animals were at rest. Subsequently, the animals were given 1% NaCl in their drinking water for 12 days, prior to a second set of blood pressure measurements after repeat arterial cannulation. Sodium intake was monitored, and was uniform among all animals. Univariate analysis of variance showed the following significant effects: (1) basal systolic blood pressure: cross ( $P < 0.0001$ ), sex ( $P < 0.0001$ ), and interaction between cross and sex ( $P < 0.05$ ); (2) basal diastolic blood pressure: cross ( $P < 0.0001$ ) and sex ( $P < 0.0002$ ); (3) sodium-loaded systolic blood pressure: sex ( $P < 0.05$ ); (4) sodium-loaded diastolic blood pressure: sex ( $P < 0.05$ ). One outlying observation for which the basal blood pressure exceeded 3 standard deviations from the sex- and cross-specific mean was removed before linkage analysis.



TABLE 1 Chromosome assignments of markers, and marker order and recombination estimates between adjacent markers for assigned linkage groups

| Chromosome | Linkage group | Marker               | Recombination distance | Chromosome | Linkage group | Marker                  | Recombination distance |
|------------|---------------|----------------------|------------------------|------------|---------------|-------------------------|------------------------|
| Chr. 1     | 1             | RatLEUKOS1           | 0.01                   | Chr. 13    |               | RatREN*                 |                        |
|            |               | RatMYL2G             | 0.01                   | Chr. 14    | 14            | Rat16FBP3               |                        |
|            |               | <i>YNH24-Ha-6</i>    | 0.02                   |            |               | <b>YNZ2-Hi-5</b>        | 0.11                   |
|            |               | <b>pASC-Ha-2</b>     | 0.03                   |            |               | <b>PER-Hi-4</b>         | 0.09                   |
|            |               | <b>YNH24-Hi-3</b>    |                        |            |               | <b>YNZ2-Ha-7</b>        | 0.02                   |
|            | 2             | RatPKCI              | 0.01                   |            |               | <b>YNZ2-Hi-1</b>        | 0.02                   |
|            |               | 2B1                  | 0.06                   |            |               | <b>PER-Hi-1 (4)</b>     | 0.00                   |
|            |               | RatCY45E1            | 0.09                   |            |               | <i>YNZ2-Hi-13 (5)</i>   | 0.30                   |
|            |               | RatKALA              | 0.00                   |            |               | <i>YNZ2-Ha-14</i>       | 0.15                   |
|            |               | RatTON2              |                        |            |               | RatALBZA*               | 0.00                   |
| Chr. 2     | 3             | RatCARB08            | 0.23                   |            |               | RatAFPGA*               | 0.02                   |
|            |               | <b>pASC-Hi-3</b>     | 0.06                   |            |               | RatCASG1                |                        |
|            |               | RatP9KA              | 0.01                   | Chr. 16    | 15            | <i>pASC-Ha-6</i>        | 0.13                   |
|            |               | RatPKLG*             |                        |            |               | RatMABPA2               |                        |
| Chr. 3     | 4             | RatCATL              | 0.20                   | Chr. 17    | 16            | <i>YNH24-Ha-4</i>       | 0.00                   |
|            |               | M22254               |                        |            |               | <i>YNH24-Hi-7</i>       | 0.08                   |
|            | 5             | RatPECG6             | 0.12                   |            |               | RatPRLSD1*              | 0.09                   |
|            |               | RatSVPIIA            |                        |            |               | F4                      | 0.08                   |
| Chr. 4     | 6             | <i>YNZ2-Hi-21</i>    | 0.22                   |            |               | RatRMC                  |                        |
|            |               | <b>YNZ22-Hi-1</b>    | 0.00                   | Chr. 18    | 17            | <i>AW-Ha-13</i>         | 0.07                   |
|            |               | RatEN3               | 0.05                   |            |               | <i>YNZ2-Hi-19</i>       | 0.20                   |
|            |               | RatA2MAC             | 0.26                   |            |               | <b>PER-Hi-5</b>         | 0.09                   |
|            |               | RatSPR               |                        |            |               | RatTGCR                 | 0.10                   |
| Chr. 5     |               | A8                   |                        |            |               | RatADBC                 | 0.07                   |
| Chr. 6     | 7             | D3                   | 0.07                   |            |               | M26718                  | 0.05                   |
|            |               | <i>pMV10.2-Ha-10</i> | 0.04                   |            |               | <i>pUCJ-Hi-6</i>        | 0.11                   |
|            |               | <i>pMV10.2-Hi-6</i>  | 0.14                   |            |               | <i>YNZ2-Hi-14</i>       |                        |
|            |               | <i>INS310-Hi-11</i>  |                        | Chr. 19    | 18            | X16379                  | 0.12                   |
|            | 8             | <i>pMV10.2-Hi-4</i>  | 0.00                   |            |               | <i>pUCJ-Ha-5</i>        | 0.00                   |
|            |               | <i>pMV10.2-Hi-8</i>  | 0.02                   |            |               | <b>pASC-Hi-1</b>        | 0.03                   |
|            |               | <i>pMV10.2-Hi-7</i>  | 0.01                   |            |               | RatUCPA                 | 0.00                   |
|            |               | <b>RatIGCA*</b>      | 0.00                   |            |               | <i>pASC-Hi-5 (3)</i>    | 0.00                   |
|            |               | <i>pMV10.2-Ha-11</i> | 0.05                   |            |               | <b>AW-Hi-2</b>          | 0.01                   |
|            |               | RatCKBR              |                        |            |               | <i>AW-Ha-1</i>          | 0.00                   |
| Chr. 7     |               | E5                   |                        |            |               | <b>AW-Ha-3</b>          | 0.12                   |
| Chr. 8     | 9             | <i>pMV10.2-Ha-8</i>  | 0.02                   | Chr. 20    | 19            | RatHOXA                 |                        |
|            |               | RatPKATA2*           |                        |            |               | <i>PER-Hi-13</i>        | 0.05                   |
| Chr. 9     | 10            | <i>AW-Hi-10</i>      | 0.12                   |            |               | <b>AW-Hi-6</b>          | 0.00                   |
|            |               | RatCRYG              |                        |            |               | <i>AW-Hi-11</i>         | 0.08                   |
| Chr. 10    | 11            | <i>pASC-Hi-8</i>     | 0.08                   |            |               | <b>pMV10.2-Hi-2</b>     | 0.02                   |
|            |               | RatACE               | 0.02                   |            |               | RatTNF                  | 0.12                   |
|            |               | RatGHGP*             | 0.12                   |            |               | <i>pASC-Hi-9</i>        | 0.01                   |
|            |               | RatNGFRR             | 0.20                   |            |               | <i>pASC-Ha-7</i>        |                        |
|            |               | RatVAMP13            | 0.01                   | Chr. X     | 20            | <i>pASC-Hi-6 (2)</i>    | 0.279                  |
|            |               | RatRHL1              | 0.00                   |            |               | <b>INS310-Hi-3 (11)</b> | 0.016                  |
|            |               | RatABPG*             | 0.02                   |            |               | <i>INS310-Hi-9 (4)</i>  | 0.203                  |
|            |               | RatMHCG              |                        |            |               | <i>PER-Ha-7</i>         | 0.080                  |
| Chr. 11    | 12            | <b>EFD-Ha-1</b>      | 0.23                   |            |               | <b>PER-Ha-2 (2)</b>     | 0.141                  |
|            |               | RatMRCOX2            |                        |            |               | RatPKFBP1               | 0.014                  |
| Chr. 12    | 13            | <i>YNZ22-Ha-2</i>    | 0.15                   |            |               | <i>PER-Hi-12</i>        |                        |
|            |               | RatLEUKOS2           | 0.07                   |            |               |                         |                        |
|            |               | H4                   | 0.05                   |            |               |                         |                        |
|            |               | <b>YNZ2-Ha-4</b>     | 0.02                   |            |               |                         |                        |
|            |               | <b>pMV10.2-Ha-3</b>  |                        |            |               |                         |                        |

Forty-nine microsatellite markers were assigned to chromosomes in a panel of somatic cell hybrids<sup>37</sup> and by linkage using the LINKAGE programs<sup>38</sup>. Nine had previously been assigned (indicated by \*) as described in ref. 39. The assignment of the renin gene was obtained from ref. 17. Markers were assigned to the same linkage group when the maximum pairwise lod score exceeded 4. The loci were divided into 28 linkage groups (20 assigned to chromosomes) containing two or more markers, and 51 singleton markers that were unlinked to other loci. Markers within the same linkage group were ordered with the GMS program<sup>40</sup>. Microsatellite loci are indicated by the sequence name from GENBANK for markers developed from known genes; otherwise we give the clone name. Minisatellite bands are designated by the probe and enzyme name, followed by the band number; bands present in SHRSP are indicated in italics, while those present in WKY are indicated in bold. In some instances, when several bands present in the same strain have nearly identical localizations (recombination frequency <2%), only one band is named in the table; a superscript number indicates the total number of bands in these cases. PCR and electrophoresis conditions for chromosome assignments were as follows: Amplifications were performed in a volume of 25 µl with final concentrations for each reagent being: 10 mM of dNTPs (Pharmacia); 0.4 µM of each primer and 0.6 U of Taq DNA polymerase (Promega). 100 ng of genomic DNA was used in each reaction. Forty cycles of PCR were carried out under the following conditions using a LEP PREM III DNA Thermal Cycler: one min at 94 °C, one min at 55 °C and 30 sec at 72 °C, preceded by an initial denaturation step (3 min at 94 °C) and followed by a final elongation step (3 min at 72 °C). PCR products were resolved on 4% agarose gel electrophoresis (3% NuSieve, 1% Sigma Type II agarose) and stained by ethidium bromide.

TABLE 2 ANOVA of selected autosomal markers (a) and of X-linked markers in females (b), with genetic analysis (c)

| (a)                   |         | Statistics by genotype (mm Hg) |                              |                              | Analysis of variance                   |                                       |                                                |
|-----------------------|---------|--------------------------------|------------------------------|------------------------------|----------------------------------------|---------------------------------------|------------------------------------------------|
| Phenotype             | Marker  | SP/SP                          | SP/WKY                       | WKY/WKY                      | ANOVA<br>all data                      | ANOVA<br>trimmed                      | Kruskal-<br>Wallis                             |
| Basal systolic*       | RatNGFR | 151.7 ± 1.8<br><i>n</i> = 31   | 150.1 ± 1.5<br><i>n</i> = 42 | 143.0 ± 1.8<br><i>n</i> = 26 | <b><i>F</i><sub>2,87</sub> = 5.5</b>   | <b><i>F</i><sub>2,57</sub> = 4.9</b>  | <b><i>X</i><sub>2</sub><sup>2</sup> = 10.3</b> |
|                       | RatGHP  | 150.4 ± 2.0<br><i>n</i> = 21   | 150.7 ± 1.4<br><i>n</i> = 59 | 144.5 ± 1.6<br><i>n</i> = 27 | <i>F</i> <sub>2,95</sub> = 3.7         | <i>F</i> <sub>2,63</sub> = 4.0        | <i>X</i> <sub>2</sub> <sup>2</sup> = 6.8       |
|                       | RatACE  | 150.7 ± 1.9<br><i>n</i> = 21   | 151.2 ± 1.5<br><i>n</i> = 49 | 145.7 ± 1.6<br><i>n</i> = 28 | <i>F</i> <sub>2,86</sub> = 2.9         | <i>F</i> <sub>2,56</sub> = 2.9        | <i>X</i> <sub>2</sub> <sup>2</sup> = 4.7       |
| Basal diastolic*      | RatNGFR | 106.3 ± 1.5<br><i>n</i> = 31   | 104.2 ± 1.3<br><i>n</i> = 42 | 96.1 ± 1.7<br><i>n</i> = 26  | <b><i>F</i><sub>2,96</sub> = 6.6</b>   | <i>F</i> <sub>2,63</sub> = 5.8        | <b><i>X</i><sub>2</sub><sup>2</sup> = 12.4</b> |
|                       | RatGHP  | 105.0 ± 1.2<br><i>n</i> = 21   | 104.1 ± 1.6<br><i>n</i> = 59 | 97.4 ± 1.6<br><i>n</i> = 27  | <b><i>F</i><sub>2,95</sub> = 6.6</b>   | <i>F</i> <sub>2,63</sub> = 5.8        | <b><i>X</i><sub>2</sub><sup>2</sup> = 12.4</b> |
|                       | RatACE  | 104.0 ± 1.7<br><i>n</i> = 21   | 105.8 ± 1.3<br><i>n</i> = 49 | 98.9 ± 1.5<br><i>n</i> = 28  | <b><i>F</i><sub>2,86</sub> = 5.1</b>   | <i>F</i> <sub>2,56</sub> = 4.6        | <b><i>X</i><sub>2</sub><sup>2</sup> = 9.4</b>  |
| NaCl-loaded systolic  | RatNGFR | 177.5 ± 2.3<br><i>n</i> = 31   | 180.0 ± 1.8<br><i>n</i> = 41 | 166.7 ± 2.1<br><i>n</i> = 27 | <b><i>F</i><sub>2,96</sub> = 10.6</b>  | <b><i>F</i><sub>2,66</sub> = 9.6</b>  | <b><i>X</i><sub>2</sub><sup>2</sup> = 18.6</b> |
|                       | RatGHP  | 176.9 ± 2.6<br><i>n</i> = 21   | 178.9 ± 1.6<br><i>n</i> = 58 | 164.8 ± 2.2<br><i>n</i> = 28 | <b><i>F</i><sub>2,104</sub> = 13.4</b> | <b><i>F</i><sub>2,72</sub> = 11.2</b> | <b><i>X</i><sub>2</sub><sup>2</sup> = 20.6</b> |
|                       | RatACE  | 177.2 ± 2.5<br><i>n</i> = 21   | 179.8 ± 1.8<br><i>n</i> = 49 | 165.9 ± 2.4<br><i>n</i> = 29 | <b><i>F</i><sub>2,96</sub> = 11.6</b>  | <b><i>F</i><sub>2,66</sub> = 10.7</b> | <b><i>X</i><sub>2</sub><sup>2</sup> = 18.4</b> |
| NaCl-loaded diastolic | RatNGFR | 117.9 ± 2.2<br><i>n</i> = 31   | 120.8 ± 1.8<br><i>n</i> = 41 | 109.3 ± 2.2<br><i>n</i> = 27 | <b><i>F</i><sub>2,96</sub> = 8.0</b>   | <b><i>F</i><sub>2,66</sub> = 6.4</b>  | <b><i>X</i><sub>2</sub><sup>2</sup> = 14.0</b> |
|                       | RatGHP  | 117.2 ± 2.5<br><i>n</i> = 21   | 120.4 ± 1.5<br><i>n</i> = 58 | 106.6 ± 2.2<br><i>n</i> = 28 | <b><i>F</i><sub>2,104</sub> = 13.7</b> | <b><i>F</i><sub>2,72</sub> = 11.5</b> | <b><i>X</i><sub>2</sub><sup>2</sup> = 21.5</b> |
|                       | RatACE  | 117.6 ± 2.5<br><i>n</i> = 21   | 121.1 ± 1.6<br><i>n</i> = 49 | 107.8 ± 2.3<br><i>n</i> = 29 | <b><i>F</i><sub>2,96</sub> = 11.8</b>  | <b><i>F</i><sub>2,66</sub> = 11.2</b> | <b><i>X</i><sub>2</sub><sup>2</sup> = 18.8</b> |

| (b)             |                                    | FEMALES                                  |                                   |                                   |                              | Analysis of variance                  |                                        |                                                |
|-----------------|------------------------------------|------------------------------------------|-----------------------------------|-----------------------------------|------------------------------|---------------------------------------|----------------------------------------|------------------------------------------------|
| Phenotype       | Marker                             | Statistics by genotype and cross (mm Hg) |                                   |                                   |                              | ANOVA<br>all data                     | ANOVA<br>trimmed                       | Kruskal-<br>Wallis                             |
|                 |                                    | WKY male × SHRSP female<br>SP/SP         | WKY female × SHRSP male<br>SP/WKY | WKY female × SHRSP male<br>SP/WKY | WKY/WKY                      |                                       |                                        |                                                |
| Basal systolic  | RatPKFBP1                          | 140.0 ± 2.6<br><i>n</i> = 11             | 145.8 ± 1.7<br><i>n</i> = 15      | 146.9 ± 4.2<br><i>n</i> = 8       | 152.2 ± 2.7<br><i>n</i> = 13 | <b><i>F</i><sub>2,44</sub> = 5.7</b>  | <b><i>F</i><sub>2,30</sub> = 14.09</b> | <b><i>X</i><sub>2</sub><sup>2</sup> = 9.6</b>  |
|                 | <b>PER-Ha-2</b><br><i>PER-Ha-7</i> | 138.2 ± 1.9<br><i>n</i> = 16             | 147.6 ± 1.9<br><i>n</i> = 12      | 145.8 ± 2.3<br><i>n</i> = 10      | 153.6 ± 3.1<br><i>n</i> = 13 | <b><i>F</i><sub>2,48</sub> = 12.5</b> | <b><i>F</i><sub>2,33</sub> = 18.7</b>  | <b><i>X</i><sub>2</sub><sup>2</sup> = 15.1</b> |
| Basal diastolic | RatPKFBP1                          | 95.5 ± 2.1<br><i>n</i> = 11              | 97.5 ± 1.8<br><i>n</i> = 15       | 98.9 ± 3.6<br><i>n</i> = 8        | 100.1 ± 1.8<br><i>n</i> = 13 | <i>F</i> <sub>2,44</sub> = 1.2        | <i>F</i> <sub>2,30</sub> = 1.0         | <i>X</i> <sub>2</sub> <sup>2</sup> = 1.9       |
|                 | <b>PER-Ha-2</b><br><i>PER-Ha-7</i> | 93.1 ± 1.8<br><i>n</i> = 16              | 99.8 ± 1.5<br><i>n</i> = 12       | 96.1 ± 1.9<br><i>n</i> = 10       | 101.8 ± 2.2<br><i>n</i> = 13 | <b><i>F</i><sub>2,48</sub> = 5.8</b>  | <b><i>F</i><sub>2,33</sub> = 5.1</b>   | <b><i>X</i><sub>2</sub><sup>2</sup> = 8.0</b>  |

|                       |                                    | MALES                                    |                              |                               |                              | Analysis of variance (2nd cross)      |                                       |                                                |
|-----------------------|------------------------------------|------------------------------------------|------------------------------|-------------------------------|------------------------------|---------------------------------------|---------------------------------------|------------------------------------------------|
| Phenotype             | Marker                             | Statistics by genotype and cross (mm Hg) |                              |                               |                              | ANOVA<br>all data                     | ANOVA<br>trimmed                      | Kruskal-<br>Wallis                             |
|                       |                                    | WKY male × SHRSP female<br>SP            | WKY female<br>WKY            | WKY female × SHRSP male<br>SP | WKY                          |                                       |                                       |                                                |
| NaCl-loaded systolic  | RatPKFBP1                          | 175.6 ± 2.3<br><i>n</i> = 15             | 176.4 ± 4.3<br><i>n</i> = 10 | 169.1 ± 3.1<br><i>n</i> = 17  | 183.6 ± 2.6<br><i>n</i> = 16 | <b><i>F</i><sub>1,31</sub> = 12.5</b> | <b><i>F</i><sub>1,21</sub> = 22.6</b> | <b><i>X</i><sub>1</sub><sup>2</sup> = 11.4</b> |
|                       | <b>PER-Ha-2</b><br><i>PER-Ha-7</i> | 177.3 ± 2.7<br><i>n</i> = 18             | 173.6 ± 3.1<br><i>n</i> = 8  | 168.8 ± 3.5<br><i>n</i> = 15  | 183.5 ± 2.7<br><i>n</i> = 19 | <b><i>F</i><sub>1,32</sub> = 11.4</b> | <b><i>F</i><sub>1,22</sub> = 16.3</b> | <b><i>X</i><sub>1</sub><sup>2</sup> = 10.1</b> |
| NaCl-loaded diastolic | RatPKFBP1                          | 119.5 ± 2.6<br><i>n</i> = 15             | 118.1 ± 4.5<br><i>n</i> = 10 | 110.8 ± 2.52<br><i>n</i> = 17 | 123.1 ± 2.5<br><i>n</i> = 16 | <b><i>F</i><sub>1,31</sub> = 12.2</b> | <b><i>F</i><sub>1,21</sub> = 20.5</b> | <b><i>X</i><sub>1</sub><sup>2</sup> = 11.4</b> |
|                       | <b>PER-Ha-2</b><br><i>PER-Ha-7</i> | 120.0 ± 2.8<br><i>n</i> = 18             | 117.0 ± 3.8<br><i>n</i> = 8  | 110.9 ± 3.0<br><i>n</i> = 15  | 123.2 ± 2.7<br><i>n</i> = 19 | <b><i>F</i><sub>1,32</sub> = 9.4</b>  | <b><i>F</i><sub>1,23</sub> = 11.6</b> | <b><i>X</i><sub>1</sub><sup>2</sup> = 9.5</b>  |



TABLE 2—continued

| (c) Results from genetic analysis of selected markers |                              |                   |                            |                |           |
|-------------------------------------------------------|------------------------------|-------------------|----------------------------|----------------|-----------|
| Phenotype                                             | Marker (chromosome)          | Data              | LR for linkage ( $X_1^2$ ) | $\hat{\theta}$ | $V_g/V_t$ |
| Basal systolic                                        | RatNGFRR (10)                | All               | <b>12.3</b>                | 0.00           | 0.12      |
|                                                       | RatGHP (10)                  | All               | <b>7.2</b>                 | 0.00           | 0.07      |
|                                                       | RatACE (10)                  | All               | 4.8                        | 0.00           | 0.05      |
|                                                       | RatPKFBP1 (X)                | Females           | <b>6.8</b>                 | 0.24           | 0.65      |
|                                                       | <b>PER-Ha-2/PER-Ha-7 (X)</b> | Females           | <b>13.8</b>                | 0.14           | 0.66      |
|                                                       |                              |                   |                            |                |           |
| Basal diastolic                                       | RatNGFRR (10)                | All               | <b>19.4</b>                | 0.00           | 0.17      |
|                                                       | RatGHP (10)                  | All               | <b>13.9</b>                | 0.00           | 0.12      |
|                                                       | RatACE (10)                  | All               | <b>9.3</b>                 | 0.00           | 0.08      |
|                                                       | RatPKFBP1 (X)                | Females           | 1.5                        | 0.44           | 0.56      |
|                                                       | <b>PER-Ha-2/PER-Ha-7 (X)</b> | Females           | <b>7.7</b>                 | 0.23           | 0.52      |
|                                                       |                              |                   |                            |                |           |
| NaCl-loaded systolic                                  | RatNGFRR (10)                | All               | <b>14.2</b>                | 0.00           | 0.20      |
|                                                       | RatGHP (10)                  | All               | <b>24.6</b>                | 0.00           | 0.22      |
|                                                       | RatACE (10)                  | All               | <b>20.1</b>                | 0.00           | 0.19      |
|                                                       | RatPKFBP1 (X)                | Males (2nd cross) | <b>9.0</b>                 | 0.00           | 0.29      |
|                                                       | <b>PER-Ha-2/PER-Ha-7 (X)</b> | Males (2nd cross) | 5.9                        | 0.00           | 0.22      |
|                                                       |                              |                   |                            |                |           |
| NaCl-loaded diastolic                                 | RatNGFRR (10)                | All               | <b>14.2</b>                | 0.00           | 0.15      |
|                                                       | RatGHP (10)                  | All               | <b>23.9</b>                | 0.00           | 0.22      |
|                                                       | RatACE (10)                  | All               | <b>20.0</b>                | 0.00           | 0.18      |
|                                                       | RatPKFBP1 (X)                | Males (2nd cross) | <b>9.1</b>                 | 0.16           | 0.56      |
|                                                       | <b>PER-Ha-2/PER-Ha-7 (X)</b> | Males (2nd cross) | 5.5                        | 0.26           | 0.47      |
|                                                       |                              |                   |                            |                |           |

a, RatNGFRR, RatGHP, and RatACE loci on chromosome 10; b, RatPKFBP1 and **PER-Ha-2/PER-Ha-7** loci on chromosome X; c, results of genetic analyses. In a, the blood pressure values have been adjusted for sex and cross to have means equal to males from the SHRSP female X WKY male progenitors. Statistics are indicated in bold when the corresponding  $P$  value was  $<0.01$ , and underlined when the  $P$  value was  $<0.0001$ . Certain parameter estimates from the linkage analysis are indicated in c;  $V_g$  represents the variance due to the major gene;  $V_t$  is the total variance. LR stands for the likelihood ratio test of linkage. Results are reported for a dominant effect of the alleles for SHRSP at *BP/SP-1*, and codominant effects at *BP/SP-2*. Statistical analysis was undertaken with the LINKAGE<sup>38</sup> and BMDP<sup>41</sup> program packages. The effects of extreme observations and distribution assumptions were evaluated by ANOVA with 15% trimming and a rank test (Kruskal–Wallis). Basal blood pressure measurements were adjusted for cross and sex before genetic analysis for autosomal markers, and the Kruskal–Wallis test was applied to adjusted values. The variances attributable to *BP/SP-1* and *BP/SP-2* loci were evaluated with standard quantitative genetic techniques<sup>42</sup> from the marker showing the strongest evidence of linkage. Closely linked minisatellite loci present in different parents have been considered simultaneously in the analyses of X-chromosome markers to maximize linkage information; in males, the results are given for **PER-Ha-2**.

\* Means in each group have been adjusted to equal those of males from the 1st cross for linkage analysis.

### ACE gene linked to *BP/SP-1*

RatGHP and RatNGFRR are microsatellite markers of the growth hormone and fast nerve growth factor receptor (NGFR) genes respectively; these genes form part of a conserved linkage group on chromosome 11 in the mouse<sup>29</sup>, and on human chromosome 17q (ref. 30). The gene for angiotensin I-converting enzyme, which plays a major part in the metabolism of vasoactive peptides, is a candidate gene for primary hypertension; it has been mapped in man to band 17q23 by *in situ* hybridization<sup>8</sup>. We therefore investigated the location of *ACE* and its linkage to blood pressure in SHRSP.

The murine *ACE* sequence was used to select primers that successfully detected a polymorphism in the WKY and SHRSP strains (see legend to Fig. 3). Linkage analysis of the  $F_2$  population placed RatACE outside the interval defined by RatGHP and RatNGFRR, at 2% recombination distance from the former (Table 1). The RatACE locus was also significantly linked to blood pressure measurements (Table 2). From multilocus analysis, the RatACE gene was within odds of 10:1 of the maximum likelihood placements for NaCl-loaded blood pressure phenotypes, and within 120:1 odds for baseline blood pressure phenotypes (Fig. 3).

### Sex-linked markers

As shown in Fig. 1, sexes of both progenitors and  $F_2$  animals have a significant effect on blood pressure. On average, basal blood pressures in  $F_2$  males from the second cross are 17 mm Hg (systolic) and 10 mm Hg (diastolic) higher than those in males from the first cross. In SHR  $\times$  WKY, a similar observation has been interpreted as evidence for the presence of a Y-linked gene influencing blood pressure<sup>6</sup>. But the sex effect in our data cannot be due only to the Y chromosome, as females also had higher basal blood pressures in the second cross (Fig. 1). As  $F_2$  females are  $X_sX_s$  or  $X_sX_w$  in cross 1, and  $X_sX_w$  or  $X_wX_w$  in cross 2, these differences pointed to the possible effects of genes on the X chromosome, which led us to examine linkage to X-chromosome markers.

To avoid possible confounding effects of the Y chromosome, the tests of X linkage were conducted separately in females, in males from cross 1, and in males from cross 2. Significant linkage was observed between baseline systolic blood pressure in females and two closely linked loci, **PER-Ha-2** and *PER-Ha-7* (see Table 1 for an explanation of the nomenclature for minisatellite bands). This suggested the presence of a second locus (denoted *BP/SP-2*) affecting blood pressure on the X

chromosome. Another marker from the same region of the X chromosome also showed cosegregation with the phenotype (Table 2).

The highest blood pressures were observed in females homozygous for the X chromosome marker alleles from WKY, with intermediate values in heterozygotes. Mean basal blood pressure did not differ significantly between heterozygotes from the two crosses. The hypertensive effect of the allele from WKY is compatible with the observation of higher basal systolic blood pressure in females from the second cross (which contains the  $X_wX_w$  homozygotes) compared to females from the first cross (which contains the  $X_sX_s$  homozygotes).

On the basis of the quantitative genetic analysis, we judged that *BP/SP-2* could account for 64% of the total variance (21 mg Hg difference between homozygote means) of basal systolic blood pressure in females. A large percentage of the variance is attributed to *BP/SP-2* because it accounts for all the difference observed between females from the two crosses under the genetic model. This interpretation is supported by other statistical analyses: the cross effect was not significant once the X-chromosome marker was included in the ANOVA ( $P > 0.25$ ), whereas the effect of the marker was significant with cross included ( $P < 0.004$ ).

The non-zero recombination estimate between basal systolic blood pressure and *PER-Ha-2/PER-Ha-7* led us to inspect the data for possible recombinants. We identified one animal with

low systolic blood pressure that had inherited two WKY alleles at *PER-Ha-2/PER-Ha-7*, and non-recombined WKY/WKY X chromosomes at the surrounding markers. If *BP/SP-2* is located near *PER-Ha-2/PER-Ha-7*, this could represent a double recombination event. Alternatively, the low systolic blood pressure in this animal could be due to effects of other loci, or the conditions under which the blood pressure measurements were obtained. Multilocus analysis gave odds of 240:1 in favour of linkage to systolic blood pressure with this observation included, and 8,600:1 in favour of linkage after it was removed (Fig. 4).

Male animals showed no evidence of X linkage for the baseline measurements, although elevated blood pressure after NaCl loading was associated with the allele from WKY at chromosome X markers in the second cross ( $P < 0.0001$  in the trimmed ANOVA; see Table 2). Significant additive effects of markers on chromosomes X and 10 were found in a 2-way ANOVA, in which they accounted for >40% of the phenotype variation in this subgroup. Blood pressures after NaCl loading were not correlated with sex-linked markers in males from the first cross.

## Discussion

We have constructed and applied a genetic linkage map from data on SHRSP  $\times$  WKY crosses, and obtained localizations for two genes, or groups of genes that may play an important role in SHRSP blood pressure in  $F_2$  cohorts. Our results illustrate

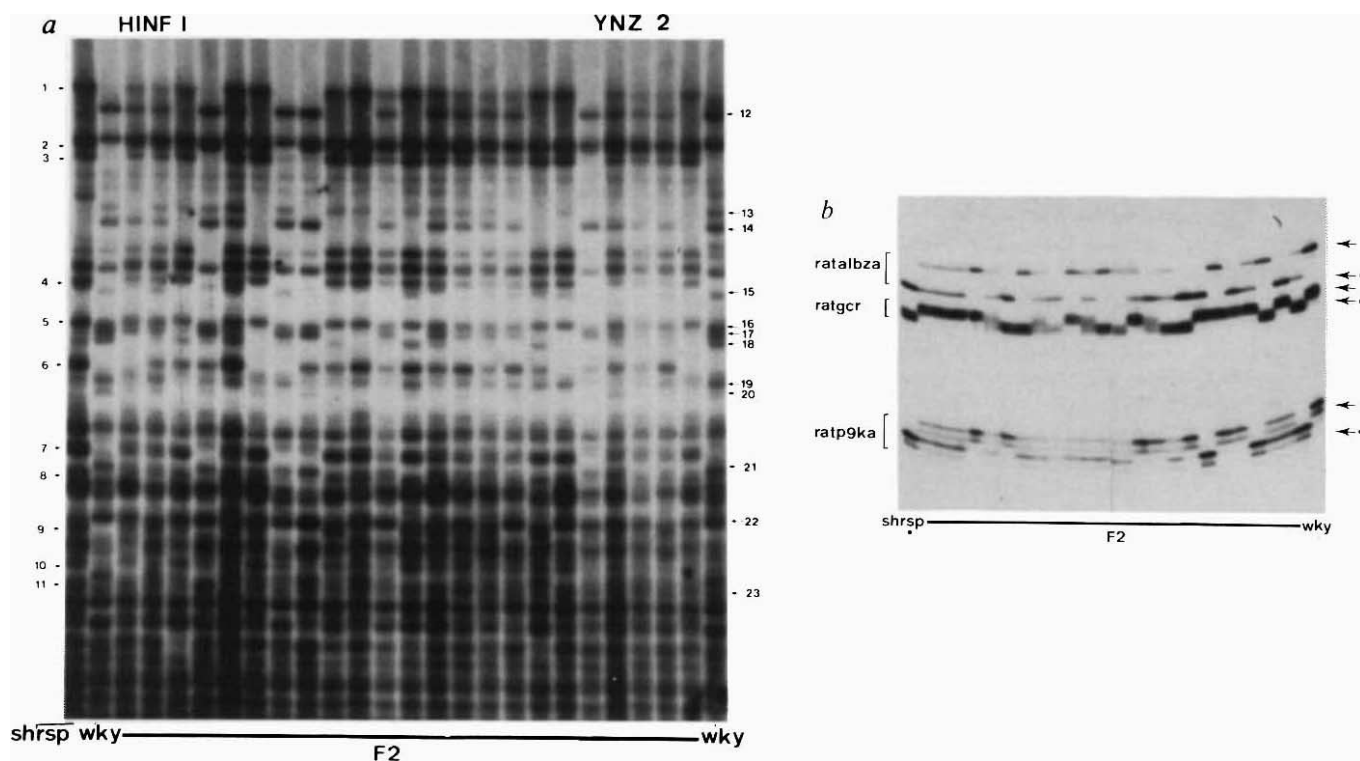


FIG. 2 *a*, *HinfI* DNA fingerprint obtained with probe YNZ2. Scored fingerprint bands are marked 1 to 11 (SHRSP) and 12 to 23 (WKY). Central lanes correspond to  $F_2$  individuals. *b*, Multiplex genotyping of three microsatellite systems: RATALBZA, RATGCR and RATP9KA. Complete PCR primer data are available on request. Arrows indicate alleles from SHRSP (●) and WKY (○). METHODS. DNA fingerprints were obtained using the following probes: PER, pMV9.17, pYNZ2, pAW101; pYNH24, pUCJ, pPHMV10.2, pINS310, pEFD134.7, pYNZ22, pASC, pMV9.17 and pPHMV10.2 are new probes isolated by screening of a mouse genomic library with pYNH37.3 and pYNZ22. Bands that deviated significantly from Mendelian segregation were removed prior to linkage analysis. Up to three non-overlapping microsatellite alleles were co-amplified in the following conditions: 75 mM KCl, 15 mM Tris-HCl, pH 8.4, 2 mM MgCl<sub>2</sub>, 0.015% gelatin, 300  $\mu$ M of each dNTP and 1  $\mu$ M of each primer.

Samples were denatured for 5 min at 95 °C and cycled 30 times through the following steps: 1 min at 93 °C, 2 min at 55 °C, 60 °C or 65 °C, then 2 min at 72 °C. All PCR were 15- $\mu$ l reactions performed in 96-microwell plates. Samples were denatured at 95 °C for 5 min in 50% formamide and 10 mM EDTA, and loaded onto 7% polyacrylamide gels containing 32% formamide, 8 M urea, 135 mM Tris-HCl, 45 mM boric acid and 2.5 mM EDTA, pH 8.3. Random microsatellites: Genomic DNA was digested to completion with *HaeIII* or *MboI*. Size fractions between 250 and 500 bp were ligated into the bacterial alkaline phosphatase dephosphorylated *AccI* and *BamHI* sites of pUC18, cloned into DH5A cells (BRL), and screened with an end-labelled (AC)<sub>15</sub> oligonucleotide. Plasmid DNA from positive clones was sequenced and primers flanking were selected using the OLIGO program<sup>44</sup>.



that mapped DNA markers can be applied directly to blood pressure phenotypes for investigations of linkage in genetic models of hypertension. Direct linkage to blood pressure, rather than to physiological variables that may be modified as a consequence of hypertension, provides evidence that the localized genes are causally related to the disease.

The *BP/SP-1* locus on chromosome 10 accounts for more than 20% of the variance of blood pressure after NaCl-loading in the  $F_2$  cohort, and a smaller proportion (12–17%) of the variance of basal blood pressure adjusted for sex and cross. Although SHRSP and SHR are not usually studied as models of NaCl-dependent hypertension, both strains show significant elevations in blood pressure in response to dietary NaCl-loading, whereas WKY shows little or no response<sup>19–23</sup>. The balance between arterial pressure and sodium intake, which is considered as a major long-term determinant of blood pressure, is altered in the SHR strain<sup>31,32</sup>, therefore, linkage to both NaCl-loaded and basal blood pressure might indicate a role of *BP/SP-1* in the development of hypertension through effects on renal function and volume homeostasis. The smaller effect of *BP/SP-1* on basal blood pressure could be due to greater experimental variation in the determination of these phenotypes; alternatively,

basal blood pressure could be influenced by a large number of genes.

We identified *ACE* as a candidate gene by comparison of the rat and human genetic maps, localizing it to the *BP/SP-1* region of chromosome 10. *ACE* is responsible for converting angiotensin I into the vasoconstrictor and aldosterone-stimulating peptide angiotensin II, and inactivates the vasodilator peptide bradykinin<sup>9</sup>. *ACE* may have a regulatory role on angiotensin II generation in plasma, as well as in tissues. In the kidney, the level of angiotensin II modulates sodium excretion<sup>33</sup>, which could provide a possible explanation of the linkage between the *ACE* locus and NaCl-induced blood pressure values found here.

One advantage of the linkage approach is that information from several markers can be combined in multilocus linkage analysis to obtain a more precise estimate for the location of a gene such as *BP/SP-1*. From such an analysis of our data, *RatACE* falls within the region of 10:1 odds from *BP/SP-1* in the analysis of blood pressure after NaCl loading, and within the region of 100:1 odds for basal blood pressure phenotypes. Our map of chromosome 10 will allow these intervals to be narrowed with data from a replicate cross, to exclude or to confirm *RatACE* as a candidate for the *BP/SP-1* gene. Mapped

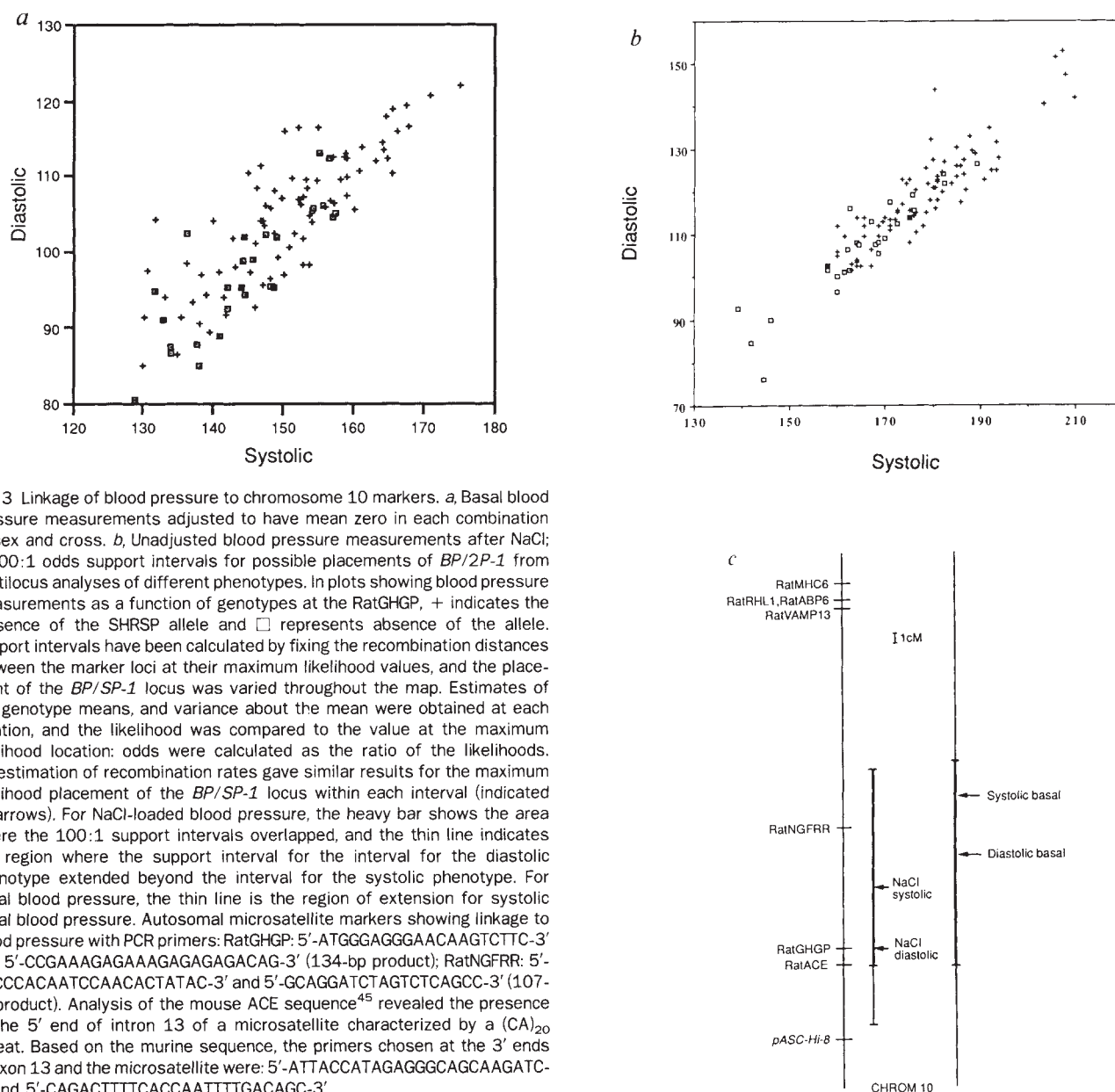
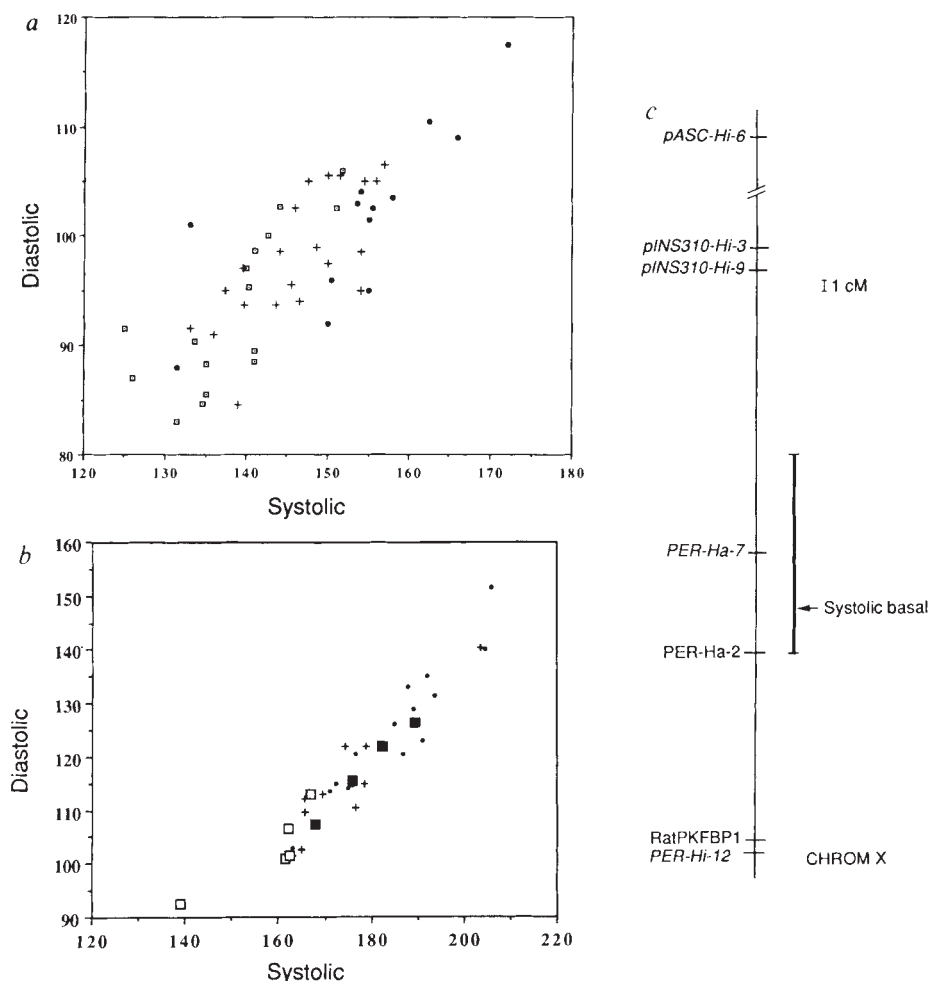


FIG. 3 Linkage of blood pressure to chromosome 10 markers. *a*, Basal blood pressure measurements adjusted to have mean zero in each combination of sex and cross. *b*, Unadjusted blood pressure measurements after NaCl; *c*, 100:1 odds support intervals for possible placements of *BP/2P-1* from multilocus analyses of different phenotypes. In plots showing blood pressure measurements as a function of genotypes at the *RatGHGP*, + indicates the presence of the SHRSP allele and □ represents absence of the allele. Support intervals have been calculated by fixing the recombination distances between the marker loci at their maximum likelihood values, and the placement of the *BP/SP-1* locus was varied throughout the map. Estimates of the genotype means, and variance about the mean were obtained at each location, and the likelihood was compared to the value at the maximum likelihood location: odds were calculated as the ratio of the likelihoods. Re-estimation of recombination rates gave similar results for the maximum likelihood placement of the *BP/SP-1* locus within each interval (indicated by arrows). For NaCl-loaded blood pressure, the heavy bar shows the area where the 100:1 support intervals overlapped, and the thin line indicates the region where the support interval for the interval for the diastolic phenotype extended beyond the interval for the systolic phenotype. For basal blood pressure, the thin line is the region of extension for systolic basal blood pressure. Autosomal microsatellite markers showing linkage to blood pressure with PCR primers: *RatGHGP*: 5'-ATGGGAGGGAACAAGTCTTC-3' and 5'-CCGAAAGAGAAAGAGAGACAG-3' (134-bp product); *RatNGFR*: 5'-ACCCCAATCCAACACTATAC-3' and 5'-GCAGGATCTAGTCTCAGCC-3' (107-bp product). Analysis of the mouse *ACE* sequence<sup>45</sup> revealed the presence at the 5' end of intron 13 of a microsatellite characterized by a (CA)<sub>20</sub> repeat. Based on the murine sequence, the primers chosen at the 3' ends of exon 13 and the microsatellite were: 5'-ATTACCATAGAGGGCAGCAAGATC-3' and 5'-CAGACTTTTACCAATTTTGACAGC-3'.

FIG. 4 Sex-linkage of female basal blood pressure phenotypes, and effects of chromosomes X and 10 on sodium-loaded blood pressures in males from cross 2: *a*, Basal blood pressure measurements in females and genotypes at the **PER-Ha-2** and **PER-Ha-7** loci; *b*, blood pressure after NaCl loading in males and alleles at the **PER-Ha-2** and RatGHP loci; *c*, 100:1 odds support interval for the placement of BP/SP-2 based on multi-locus analysis. These two markers exhibit no recombination in females, and have been combined to produce a completely informative minisatellite locus in *a*. Because blood pressure after NaCl loading showed strong effects of both chromosome X and 10 in males from cross 2, we examined composite genotypes for these loci. Genotypes are indicated as: □ for SHRSP/SHRSP; + for SHRSP/WKY; ● for WKY/WKY. Results were similar for **PER-Ha-7**, except that one recombinant was observed between the two marker loci. Genotypes are indicated as: (1) ● for WKY **PER-Ha-2** and SHRSP RatGHP alleles; (2) + for SHRSP **PER-Ha-2** and SHRSP RatGHP alleles; (3) ■ for WKY **PER-Ha-2** and absence of SHRSP RatGHP alleles; (4) □ for SHRSP **PER-Ha-2** and absence of SHRSP RatGHP alleles. Means, standard error of means, and sample size for the four genotype classes were: (1)  $184.7 \pm 3.1$  ( $n=15$ ); (2)  $179.0 \pm 4.5$  ( $n=4$ ); (3)  $175.0 \pm 3.9$  ( $n=11$ ); (4)  $158.4 \pm 4.6$  ( $n=5$ ). A two-way ANOVA in this group showed significant effects of **PER-Ha-2** on chromosome X ( $P < 0.001$ ) and RatGHP on chromosome 10 ( $P < 0.02$ ); interaction between the loci on different chromosomes was non-significant ( $P=0.29$ ). The support interval was calculated as described in Fig. 3, with one observation removed as discussed in the text.

Primers chosen for the X chromosome marker RatPKFBP1 were: 5'-AGTCTTTTGCTTCAGGGCT-3' and 5'-AGGGCTGTGGGTGGTACTAC-3' (139-bp product size).



markers can be applied in the same way to the study of other candidate genes. Co-segregation of blood pressure with a renin polymorphism has been reported in the sodium-sensitive Dahl rat<sup>16</sup>. Although a smaller effect of renin has been described in one SHR cross<sup>17</sup>, no evidence of this linkage was found in our SHRSP crosses<sup>7</sup>. We were unable to demonstrate linkage to the kallikrein gene (rat chromosome 1), or to the tumour necrosis factor gene (rat chromosome 20), in regions that have previously shown weak associations with blood pressure in other crosses<sup>34,35</sup>.

Our data support previous studies demonstrating an association of elevated blood pressure in males with the presence of the SHR Y chromosome<sup>6</sup>, and we have extended sex linkage to include the X chromosome. The BP/SP-2 locus on chromosome X may account for most of the variability of basal systolic blood pressure in female animals from our F<sub>2</sub> cohorts. The WKY alleles at the marker loci were associated with increased blood pressure, which is compatible with the observation that blood pressure is on average higher in females from the second cross. Although we observed no cosegregation of X markers with blood pressure after NaCl-loading in females or in males from the first cross, we found evidence of linkage to NaCl-loaded blood pressure in males from the second cross. These results illustrate the complex nature of genetic control of blood pressure in these experimental crosses.

The example of the BP/SP-2 locus suggests that selection for hypertension during the formation of an inbred rat strain can

also lead to the incorporation of non-hypertensive alleles at some loci involved in blood pressure regulation; indeed, this may be required for viability of the strain. Also the WKY strain has been noted to show somewhat higher blood pressure than other normotensive laboratory strains (M.V., unpublished data, and ref. 36), and the WKY allele at BP/SP-2 may be partly responsible for this increase. Although our conclusions are specific to SHRSP and WKY alleles in crosses involving these two strains, chromosomes 10 and X could be examined in other crosses to investigate whether effects of the BP/SP-1 and BP/SP-2 loci can be generalized to other models of hypertension. Comparative mapping would also suggest study of chromosomes 17q and X as candidate regions in human hypertension; no comparative data are yet available to narrow the region of interest on chromosome X.

Finally, as BP/SP-1 and BP/SP-2 do not account for all of the blood pressure variance in the F<sub>2</sub> population, other important genes are likely to contribute to SHRSP hypertension. Although in our study no other autosomal markers met the statistical criterion for linkage, genes with smaller effects, or in other areas of the genome not adequately covered by our map, may have been missed. Linkage studies using other DNA markers in these crosses, or crosses derived from other hypertensive strains, will lead to localizations of other genes involved in the regulation of blood pressure, or in the determination of related phenotypes. Our results demonstrate the random marker linkage studies represent a feasible and powerful approach to identifying genetic



loci involved in the aetiology of hypertension. Knowledge derived from these studies may ultimately lead to a better understanding of hypertension in humans, opening the possibility for improved treatment and prevention.

*Note added in proof:* While this article was in press we have

learned that another group has reached similar conclusions concerning the linkage of blood pressure to *GHP* and *ACE* in the crosses described here (Jacob *et al. Cell*, in press). K.L. and D.G. are co-authors on this paper; their contributions include performing the cross. □

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# A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules

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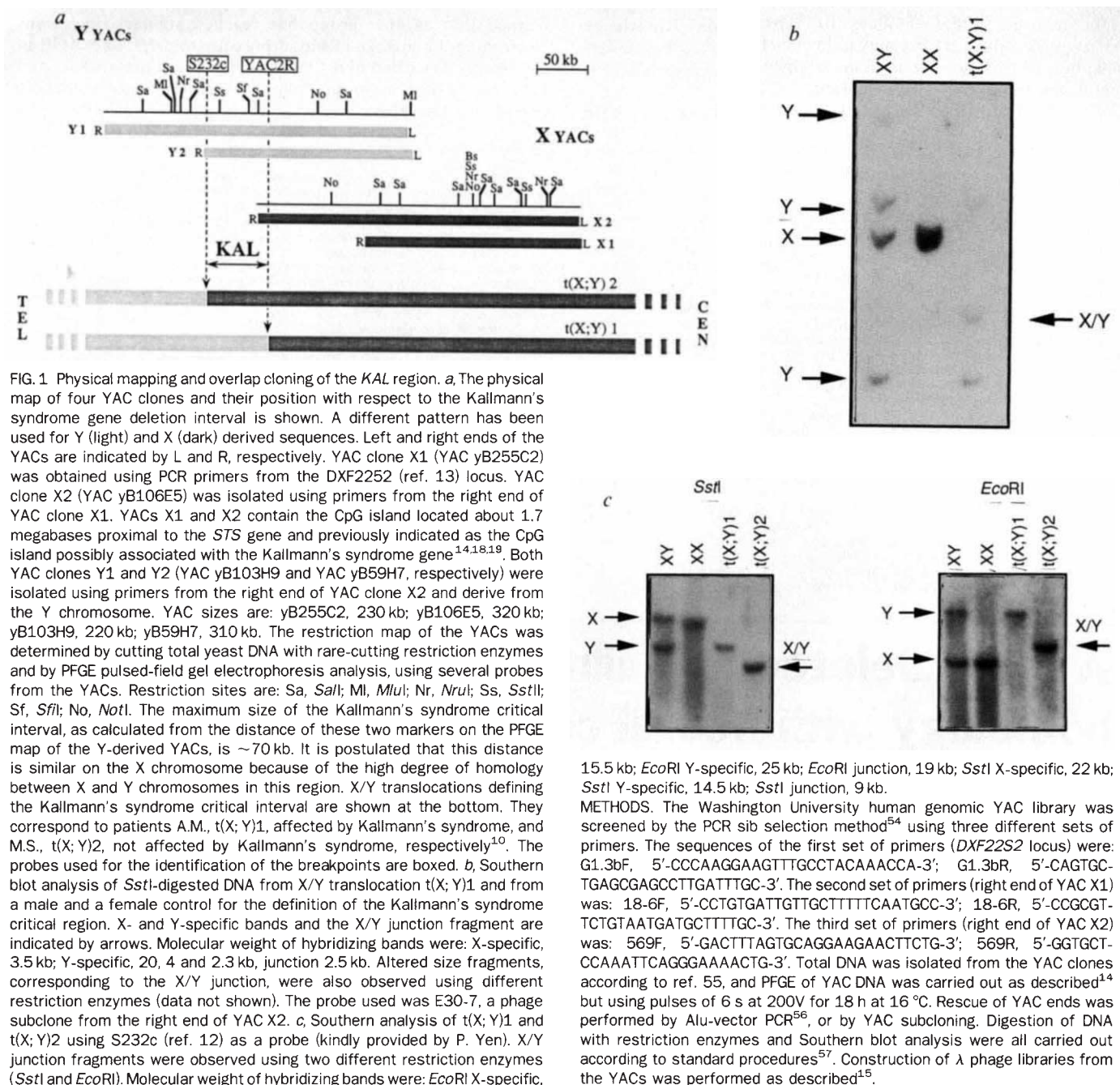
Kallmann's syndrome (clinically characterized by hypogonadotropic hypogonadism and inability to smell) is caused by a defect in the migration of olfactory neurons, and neurons producing hypothalamic gonadotropin-releasing hormone. A gene has now been isolated from the critical region on Xp22.3 to which the syndrome locus has been assigned: this gene escapes X inactivation, has a homologue on the Y chromosome, and shows an unusual pattern of conservation across species.

The predicted protein has significant similarities with proteins involved in neural cell adhesion and axonal pathfinding, as well as with protein kinases and phosphatases, which suggests that this gene could have a specific role in neuronal migration.

KALLMANN's syndrome<sup>1</sup> is a genetic disorder of both the gonadal and olfactory functions. The characteristic hypogonadotropic hypogonadism is secondary to deficiency of hypothalamic gonadotropin-releasing hormone (LH-RH) and the inability to smell (anosmia) is due to the absence of olfactory bulbs and tracts. In some patients these symptoms are associated with other neurological abnormalities, including mirror movements of hands and feet (synkinesia), uncoordinated eye movements (nystagmus), and with abnormalities in the development of the kidney (unilateral renal aplasia or hypoplasia)<sup>2–5</sup>. It has

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**FIG. 1** Physical mapping and overlap cloning of the *KAL* region. **a**, The physical map of four YAC clones and their position with respect to the Kallmann's syndrome gene deletion interval is shown. A different pattern has been used for Y (light) and X (dark) derived sequences. Left and right ends of the YACs are indicated by L and R, respectively. YAC clone X1 (YAC yB255C2) was obtained using PCR primers from the DXF2252 (ref. 13) locus. YAC clone X2 (YAC yB106E5) was isolated using primers from the right end of YAC clone X1. YACs X1 and X2 contain the CpG island located about 1.7 megabases proximal to the *STS* gene and previously indicated as the CpG island possibly associated with the Kallmann's syndrome gene<sup>14,18,19</sup>. Both YAC clones Y1 and Y2 (YAC yB103H9 and YAC yB59H7, respectively) were isolated using primers from the right end of YAC clone X2 and derive from the Y chromosome. YAC sizes are: yB255C2, 230 kb; yB106E5, 320 kb; yB103H9, 220 kb; yB59H7, 310 kb. The restriction map of the YACs was determined by cutting total yeast DNA with rare-cutting restriction enzymes and by PFGE pulsed-field gel electrophoresis analysis, using several probes from the YACs. Restriction sites are: Sa, *Sall*; Ml, *MluI*; Nr, *NruI*; Ss, *SstII*; Sf, *SfiI*; No, *NotI*. The maximum size of the Kallmann's syndrome critical interval, as calculated from the distance of these two markers on the PFGE map of the Y-derived YACs, is ~70 kb. It is postulated that this distance is similar on the X chromosome because of the high degree of homology between X and Y chromosomes in this region. X/Y translocations defining the Kallmann's syndrome critical interval are shown at the bottom. They correspond to patients A.M., t(X;Y)1, affected by Kallmann's syndrome, and M.S., t(X;Y)2, not affected by Kallmann's syndrome, respectively<sup>10</sup>. The probes used for the identification of the breakpoints are boxed. **b**, Southern blot analysis of *SstI*-digested DNA from X/Y translocation t(X;Y)1 and from a male and a female control for the definition of the Kallmann's syndrome critical region. X- and Y-specific bands and the X/Y junction fragment are indicated by arrows. Molecular weight of hybridizing bands were: X-specific, 3.5 kb; Y-specific, 20, 4 and 2.3 kb, junction 2.5 kb. Altered size fragments, corresponding to the X/Y junction, were also observed using different restriction enzymes (data not shown). The probe used was E30-7, a phage subclone from the right end of YAC X2. **c**, Southern analysis of t(X;Y)1 and t(X;Y)2 using S232c (ref. 12) as a probe (kindly provided by P. Yen). X/Y junction fragments were observed using two different restriction enzymes (*SstI* and *EcoRI*). Molecular weight of hybridizing bands were: *EcoRI* Y-specific, 15.5 kb; *EcoRI* junction, 19 kb; *SstI* X-specific, 22 kb; *SstI* Y-specific, 14.5 kb; *SstI* junction, 9 kb.

been suggested that Kallmann's syndrome should be considered as a multiple congenital anomaly with at least two developmental field defects, involving the central nervous system and the urogenital system<sup>2</sup>.

It is known from immunocytochemical studies in the mouse that neurons producing LH-RH share a common origin and migration pathway with the vomeronasal and terminalis nerves, suggesting that the symptoms of Kallmann's syndrome could be explained by a cell migration defect specifically affecting these types of neurons in the olfactory placode of the developing brain<sup>6,7</sup>. The brain of a fetus affected by Kallmann's syndrome showed absence of olfactory bulbs and tracts and no migration of LH-RH-producing neurons<sup>8</sup>. The syndrome can therefore be used as a disease model for the study of mechanisms involved in neuronal migration.

We have previously assigned the locus for the X-linked form of this disease to Xp22.3 by deletion mapping<sup>9,10</sup> and linkage analysis in families with isolated Kallmann's syndrome<sup>11</sup>. Here

we report the isolation of a gene, *KALIG-1* (Kallmann's syndrome interval gene 1), mapping to the Kallmann's syndrome critical region on the distal short arm of the human X chromosome. The *KALIG-1* gene shares homology with molecules involved in cell adhesion and axonal pathfinding, suggesting that a molecular defect in this gene causes the neuronal migration defect underlying Kallmann's syndrome.

### Isolation of the *KALIG-1* gene

As shown in Fig. 1a, the Kallmann's syndrome critical interval spans the region from the breakpoint of an X/Y-translocation patient with Kallmann's syndrome, t(X;Y)1, to the breakpoint of another X/Y translocation, t(X;Y)2, from a patient without Kallmann's syndrome. Probe E30-7 and S232c (ref. 12) recognized altered size fragments in t(X;Y)1 and t(X;Y)2 DNA, respectively (Fig. 1b and c). Therefore these probes represent landmarks for the proximal and the distal limits of the Kallmann's syndrome mapping interval on Xp22.3, respectively.



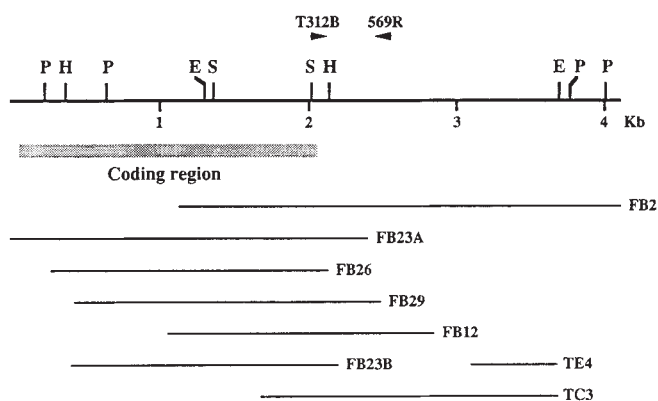


FIG. 2 Characterization of *KALIG-1* transcripts. Schematic representation of the overlapping cDNA clones corresponding to the *KALIG-1* gene with the restriction map of the consensus cDNA sequence shown at the top. The coding region is indicated by the bar. cDNA clones indicated by FB, TE and TC have been derived from human fetal brain, total embryo and teratocarcinoma cDNA libraries, respectively. E, *EcoRI*; H, *HindIII*; P, *PstI*; S, *SacI*. The positions of oligonucleotide primers T312B and 569R used for RT-PCR are also indicated.

**METHODS.** Features of cDNA libraries were: (1) male fetal brain (21 weeks) in  $\lambda$ GT11, oligo(dT)-primed (Clontech); (2) female fetal brain (17–18 weeks) in  $\lambda$ ZAPII oligo(dT) plus random-primed (Stratagene); (3) total embryo (8 weeks) in  $\lambda$ GT10 oligo(dT)-primed (custom-made by Clontech); (4) undifferentiated teratocarcinoma cell line (male) in  $\lambda$ GT10 oligo(dT)-primed (G.P.). Screening of cDNA libraries were performed as in ref. 58;  $10^6$  plaque-forming units were screened for each library. The position of the restriction sites was determined by sequence analysis (Fig. 4a).

Directional chromosome walking towards the Kallmann's syndrome locus (*KAL*) was initiated using the yeast artificial chromosome (YAC) system. Probe G1.3b (*DXF22S2*)<sup>13</sup>, previously the closest available marker to the Kallmann's syndrome locus on the proximal side<sup>14</sup>, was used as a starting point. End fragments from the YACs were used as probes on our mapping panel in order to orient the YACs with respect to the X chromosome and to the *KAL* locus (data not shown). The YAC contig shown in Fig. 1a includes four YAC clones, two of which have been assigned to the X chromosome (YACs X1 and X2), and two to a homologous region on the Y chromosome (YACs Y1 and Y2).

We have searched for evolutionarily conserved sequences within the YAC clones. YACs were subcloned in a  $\lambda$  phage vector and the overlaps between phage clones determined<sup>15</sup>.

Phage clones found to be evolutionarily conserved, by hybridization to 'zoo blots' of DNA of distantly related species (including human, cow, cat, dog, chicken, rabbit, mouse and hamster), were used for the screening of complementary DNA libraries from three different RNA sources. Several cDNA clones, corresponding to a gene from the Kallmann's syndrome region (*KALIG-1*), were isolated.

We have used the longest of the cDNA clones corresponding to the *KALIG-1* gene as a probe for the screening of a human female fetal brain cDNA library. Figure 2 illustrates the overlap of the cDNA clones isolated, as determined by restriction mapping, polymerase chain reaction (PCR) and sequence analysis. The restriction map of the consensus sequence and the position of the coding region are shown at the top of Fig. 2. By mapping both the 5' and 3' ends of the *KALIG-1* cDNA on the physical maps of the YAC contig, we could establish that the *KALIG-1* gene spans ~210 kilobases (kb) of DNA (data not shown).

### ***KALIG-1* is deleted in Kallmann patients**

Probes corresponding to both the 3' and 5' ends of *KALIG-1* were hybridized to *EcoRI*-digested DNAs from deletion and translocation cases with Kallmann's syndrome and from two translocation cases without the disease whose breakpoints are close to the Kallmann's syndrome critical interval. All the probes used detect male-specific bands, indicating the presence of sequences homologous to the *KALIG-1* gene on the Y chromosome. Probe E30-7, a genomic fragment containing part of the last exon at the 3' end of the gene, showed a deletion in all Xp22.3-deletion and-translocation cases affected by Kallmann's syndrome, whereas it was not deleted in any of the Xp22.3 deletion and translocation cases without the disease (Fig. 3a, and data not shown). Complementary DNA probes pFB26 and pFB2, spanning several exons, showed an abnormal pattern for X-specific hybridization bands in all patients with Kallmann's syndrome (Fig. 3b). A genomic probe, 29N1.7, corresponding to the 5' end of the gene, was deleted in only three of the deletion patients with Kallmann's syndrome (Fig. 3c). These data demonstrate that two X/Y translocation and two interstitial deletion breakpoints from patients with Kallmann's syndrome reside within the *KALIG-1* gene. Furthermore, the analysis of these partial deletion cases established the orientation of the *KALIG-1* gene with respect to the X short arm, with the 5' end located towards the centromere and the 3' end towards the telomere. We have not detected major rearrangements of the *KALIG-1* gene in about 20 patients with isolated (not associated with any other disorder) Kallmann's syndrome, although only nine of

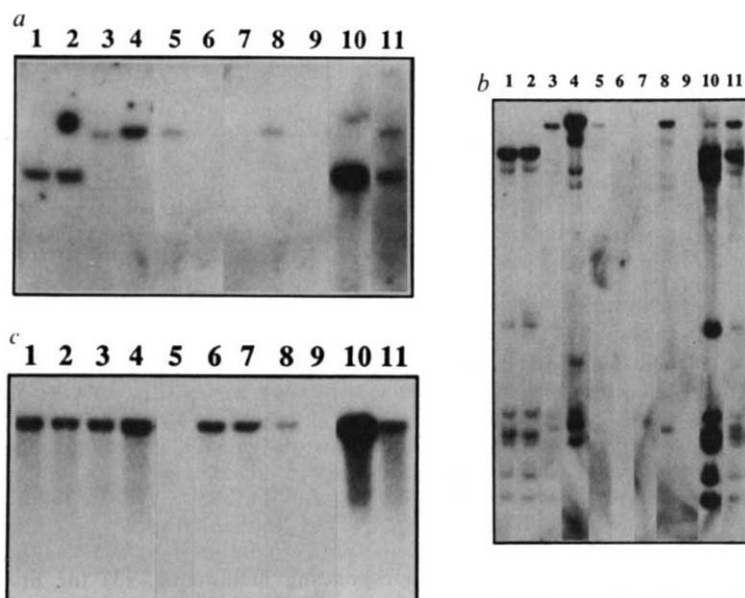


FIG. 3 Analysis of patients with Xp22.3 deletions and translocations. Lanes 1, F.L.; 2, M.S.; 3, A.M.; 4, M.B.; 5, R.C.; 6, D.S.; 7, J.G.; 8, J.D.; 9, D.N.; 10, 5 X chromosome human cell line; 11, normal male. All patients have already been described<sup>10</sup>, with the exception of M.B. (lane 4). M.B. is a patient with an X/Y translocation (Xp22.3/Yq11.2) affected by short stature, mental retardation, X-linked recessive chondrodysplasia punctata, X-linked ichthyosis and Kallmann's syndrome (S. Jeffery and L. Pereira, unpublished data). All patients with the exception of F.L. and M.S. (lanes 1 and 2) have Kallmann's syndrome. The probes used were: a, E30-7, a genomic fragment containing the last exon at the 3' end of the gene; b, pFB26 and pFB2 subclones of phage clones FB26 and FB2 (see Fig. 1); and c, 29N1.7 a genomic fragment hybridizing to the 5' end of the cDNA. All the probes used detect both X and Y specific bands. The X-specific bands can easily be recognized because their intensity is much higher in the lane corresponding to the 5 X chromosome cell line. The Y-specific bands are absent in some lanes as they correspond to human/rodent somatic cell hybrids which have lost the human Y chromosome. Restriction digestion and Southern blot analyses were all carried out according to standard procedures. Conditions for hybridizations and washings were as described<sup>10</sup>.

2136: CAGATGCTGCTGCTGCTTGGAATCTGGCGACGAGCATCTGCTCCAGAGCGACGTGG  
2195: AGACGCTGTCAGAGGCGCGGACCTCCCTATGCTGCTTTAGTGCAGGAAGACCTCTGCTG  
2254: ATCATGGACGCACTCTGGACAGCTGGAGAACACATGATGTTGGTGAACAGACACACAGCT  
2313: TCCCTCAACAGCATGGAGAAATGGAATAGGCGCTTTTAACTGCTAAATTTTGTGTTCT  
2372: TATATGGTGTCGCTCTATTTCTATGGAATTACAACAGAACTCAGTTTCCCTGAAATTTGG  
2431: AGCACTAACTCGCCCAAAAGGAGATGTTTACAAATACCAATACCACTAACACTAAGC  
2490: GATCTCTTAATCAATAAAATATTTTGCATAAAATTTAGTCTGATATGAAAAATCT  
2549: ATGAGTAATGACAGCTCTGTTTCTTTTGTGCTGCTTTCTTCTTAAGATCTTTTATCTACGT  
2608: GATTTTAAATGAAAGTTGTAATCTAAATATACCGCGAATTTAAATCTAAAAATGG  
2667: TCTCTTTTAAGTACCTGTGGCTGCTCTTTATCGCAAGGTAAATCAAGTCCCTCTTAT  
2726: AANATATGATTTCTCAAAAGACCTCCGACGAGGAACTCAATGAAATAAGCTGCCTAAT  
2785: CATATTTGACCTCTGGAGAAATAAAATTTCTTTGGGGATGCCCTTTAATTTTGTATCT  
2844: ATATGTTGAGAGTTTCTTGATATGTTATCTATTATTTATCTTCTCTATTCTCTCTCA  
2903: TCGCAATATAGCTTTTGTGCTACTTTTGTGCTACCTCTCGAAATTTGCAAAACCTTCT  
2962: TGTCTCAATGAAAAAACTCCAACTTTGTGAGCATGTTTAAATCTTTGACAGAAATTTGGC  
3021: TTCTCTCAATGCAAGTAGGCTGTTGCTGTGCTAGTATATATGGGTAAATTTTAAAAA  
3080: TTTCTTGATGTCAGAGGAAGCAATTTGTTGCTATTAAATCCACCAATATGCTCTTCA  
3139: ATCTAGTATATCTTACTTTTGTGAAATTTTATGTCGCAAAATCTGGCTCTGTGATGAT  
3198: GAATCTTAAGCTAAAAAGCTTTATCAATATAGAGTTTGGCAGTGATATTTGTAACAACT

$b$

5'  3'

The entire sequence of 4,093 base pairs (bp) of cDNA is shown in Fig. 4a. It contains a single long open reading frame that starts with an ATG at nucleotide 64 and ends with a TAA stop codon at nucleotide 2,104, giving a predicted protein product of 680 amino acids. Three lines of evidence suggest that the ATG in position 64 is the initiator codon: (1) the nucleotide sequences flanking this codon fulfill Kozak's criteria for an authentic initiation codon<sup>16</sup>; (2) hydrophobicity analysis<sup>17</sup> indicates the presence of a non-polar signal peptide of 19 amino acids following the corresponding methionine; (3) the first 200 bp of the cDNA are very rich in CpG dinucleotides and



C

## Domain A

|                  |     |                                                |     |
|------------------|-----|------------------------------------------------|-----|
| <b>KALIG-1</b>   | 131 | QGDCEPAEKASGFAAACVSEVDNECCSGVKKCCSNGCGHTCQVP   | 175 |
| <b>WDNM</b>      | 14  | EKPGKCPKNPPRSIGTCVEICSGDQSCPNIQCCSNGGHVCKSP    | 58  |
| <b>WYRT</b>      | 81  | RCPWNPQIMIAAGPCPKDNCPSIDSDCSGTMKCCNNGCIMSMDP   | 126 |
| <b>TITTOR</b>    | 60  | PPERPGVCPKTSGGPGLCHGDSDDCKEGQKCCFDGCGYGLTV     | 114 |
| <b>Elafin</b>    | 12  | KGPSPIILIRCAMLNPPNCLKDDCPGKIKCCSGSGMACFVP      | 56  |
| <b>ALK1</b>      | 94  | KPGKCPVTYGGCLMLNPPNCCSDWCCPGKKRCCPDTCGKCLDP    | 129 |
| <b>Consensus</b> |     | <u>C . . . . C . . . . C . . . . C . . . C</u> |     |

## Domain B

|                  |      |                                                       |  |
|------------------|------|-------------------------------------------------------|--|
| <b>KALIG-1</b>   | 262  | AQTDERVQLTDIFSRWYFERYAAVNVHGTTRGTAPSKHFRSSKDPSPAPP    |  |
| <b>N-CAM-L1</b>  | 666  | KVPGNQSTTLKLEFYVHYFERYTAINKYGGERSPVSESVVTPPEAAPEKN    |  |
| <b>TAG-1</b>     | 665  | IEGNAETAQVLGLMWMQYFERYSAINTLTGGERSPGSKIRTEAVPSVA      |  |
| <b>NCS-F3</b>    | 661  | IEGNMESAKAVDLIEMWEXFERYVAINTLTGGERSPGSKIRTEGAPNVA     |  |
| <b>Contactin</b> | 650  | IEGNMESARVIDLIEMWEXFERYVAINTLTGGERSPGSKIRTEGAPNVA     |  |
| <b>Twitch</b>    | 3974 | GNILDTKRRVTGLTKKTYFERYAAVNAAGGEGYSVNSVPITADNAPTRPK    |  |
| <b>LAR</b>       | 357  | DGVAATRYSIGGLSEFSEYAFERYIAVNSIGRGFPSEAVRARTGEQAPSSPP  |  |
| <b>MLCK</b>      | 721  | TTORSTSFNVQDLQADREYFERYRAANVYGISEPSQSEVVVKVGEQEEL     |  |
| <b>NRG</b>       | 666  | KVPNTDSSVVMQSEWANYFERYIAFNKIGASPEAHSDSCCTQPDVFFKN     |  |
| <b>ITB4</b>      | 1561 | PNPAQTSVVVVDLIENHSYFERYRAQSQEGMGREREGVITTESQVHPQSPL   |  |
| <b>N-CAM-1</b>   | 565  | EANMEGIVTIMGLKETRYAVLAIAINCKGLIGETSAATEFKTPVREPSAP    |  |
| <b>KROS</b>      | 18   | NGSCDSICTWKAENLEGTFFRYAAANMLIGEGYSDTSKDIVLAKDVTSP     |  |
| <b>DMSVLS</b>    | 879  | RNIRGPITFGLQRLQEDNLYQIRVRAINVDEGEPEWTEFLAARTWFLGPHRL  |  |
| <b>Consensus</b> |      | <u>L . P . . . Y . FRU . A . N . . G . GEPS . . S</u> |  |

## Domain C

|                  |      |                                                               |  |
|------------------|------|---------------------------------------------------------------|--|
| <b>KALIG-1</b>   | 601  | PNSIISQSLPSDHYVLTYPNLPRESTLYRLEYQVLTGCGEGEATIKTFRTPELPSSAHR   |  |
| <b>LAR</b>       | 446  | RRPPNAWHKNTDAGLLTYGSLLEGITYSLRYIAFTAVGGPSPFTIQVKTCQGVPAQPA    |  |
| <b>TAG-1</b>     | 858  | DNEAADRVRTAGLDSARYTGLNEMTKYHVITYRANRAGTGPASPADAMTVKPPRRFP     |  |
| <b>ROS</b>       | 986  | FSAHSKFLASEQHSPLPVTFVEGLFAYALFNLSYTPYTYWKGKTSLSLRAPETVPYAPEN  |  |
| <b>DLAR</b>      | 756  | KGFLNEPFKFDVDTLEFNITGLQEDTKYSICVIAITRKGGDRSAIVVKTGGVVRPT      |  |
| <b>FNHU</b>      | 1549 | NGPGPTTKTAGPDQTEMTEGLQETVEYVVSVAQNPSEGESQPLVQTAVTNIDRPKGLAF   |  |
| <b>ITB4</b>      | 1667 | GGPATAFRVVDGSPESRLTYPLSENVPYKFKYQARTTEGGEREGITTESQDGGFFPQ     |  |
| <b>N-CAM-L1</b>  | 862  | KRHHKSHVIVPANTISAILSLRAYSSTHYEYQAFNGRGIGPASEWTFSTPEGVPHPEA    |  |
| <b>Contactin</b> | 840  | DKEAAAQRVQVNSQYESTKLENLEKMTYRHYDYSFNSAGYGPSPRTIDITRKAPPSQRP   |  |
| <b>TKR</b>       | 484  | HVLNQDEERYQMVLPRVLLTLEQDPTTYIVKRMITPLIGGPFSPDHEFTSPSPVSRGLT   |  |
| <b>Twitch</b>    | 1411 | DLATGRNVPCGRSETTITYPNLQGHXYKFRVRAVNVKGESDPLTNTAILAKNPVEYVPG   |  |
| <b>ECK</b>       | 475  | KKGDSNSYNVRRTEGFSVTLDDLAEDTXYLVQYQALQPGAGSKVHEFQTLSPEGSGNL    |  |
| <b>DMSVLS</b>    | 505  | SAGNTRQELLPAQRTSCIFAQLQELQNYTVALTMINKQGGPSTTVSVIVTKSPLEPQQQLQ |  |
| <b>Consensus</b> |      | <u>TY . GL . P . T . Y . . . Y . A . T . . G . GP . S</u>     |  |

d

Consensus domain B: L . P . . . Y . FRU . A . N . . G . GEPS . . S  
L P Y U A G G S

Consensus domain C: TU . GL . P . T . Y . . . U . A . T . . G . GP . S

contain the rare cutter restriction sites for *Bss*HII and *Not*I corresponding to a CpG island (data not shown), which has been suggested to be associated with the 5' end of the Kallmann's syndrome gene<sup>14,18,19</sup>. No hydrophobic transmembrane domain could be identified. Six potential *N*-linked glycosylation sites are also indicated in Fig. 4a.

Analysis of the predicted protein product revealed the presence of three domains shared with other proteins (domains A, B and C). The positions of these domains in the *KALIG-1* putative protein product are schematically shown in Fig. 4b and their features shown in Fig. 4c. Domain A contains a sequence characteristic of the four-disulphide-core domain<sup>20</sup> shared by several proteins, including protease inhibitors and neurophysins. Domains B and C represent part of a previously identified fibronectin type III domain found in most cases in multiple copies in both neural cell adhesion molecules<sup>21</sup> (associated with immunoglobulin C2 domains) and in tyrosine phosphatases<sup>22</sup>. A summary of the highest scoring sequences for domains B and C is shown in Table 1. Although we derived the consensus for domains B and C independently, the comparison in Fig. 4d indicates that they are related to one another.

FIG. 4 a, Sequence analysis of the *KALIG-1* gene and of the predicted protein product (in one-letter amino-acid code). In the DNA sequence, *Eco*RI sites at the beginning and at the end of the sequence indicate *Eco*RI linkers. The position of the conserved domains is underlined. The hydrophobic region at the N terminal, probably corresponding to a leader peptide, has also been underlined (dashed). The position of six potential *N*-linked glycosylation sites is indicated by dots. The entire sequence has been deposited in GenBank, accession number X60299 *KALIG-1*. During the process of sequence assembly, the sequence of the first and last five amino acids of the *KALIG-1* predicted protein product was compared to a sequence corresponding to the same gene, isolated independently (C. Petit and J. Weissenbach, personal communication). We have noticed a discrepancy in the C-terminal region of the two sequences, due to an error in our sequence, now corrected. b, Positions of the conserved domains A, B and C in the *KALIG-1* protein. c, The consensus sequence for domain A corresponds to a previously identified four-disulphide-core domain. Some examples of the molecules containing this domain are shown. The acronyms used are decoded in Table 1. Other proteins are: WDNM (WDNM1 protein)<sup>59</sup>, WYRT (whey acid protein precursor)<sup>60</sup>, TITTOR (basic protease inhibitor)<sup>61</sup>, Elafin (elafin)<sup>62</sup>, ALK1 (antileukoproteinase 1)<sup>63</sup>. Characters identical to the consensus are highlighted in bold and underlined. The consensus sequences for domains B and C have been defined. A consensus residue must be present in at least one half of the aligned columns. Consensus characters are highlighted in bold and underlined. Conservative alternatives (as defined by the amino-acid class hierarchy in ref. 64) to the consensus are highlighted in bold only. d, Conserved amino acids between the consensus of domains B and C.

METHODS.  $\lambda$  cDNA clones were subcloned in plasmids using the  $\lambda$ ZAPII plasmid rescue procedure according to the manufacturer's specifications (Stratagene) or by standard techniques. All nucleotide sequences were determined automatically using an Applied Biosystem ABI 370A fluorescent sequencer, as described by Gibbs *et al.*<sup>65</sup>, and manually

using a Sequenase sequencing kit (USB) on both single- and double-stranded templates. Assembly of DNA sequences and search for open reading frames were performed using the computer program DNA Strider<sup>66</sup>. Sequence comparison was performed using the facilities of the Molecular Biology Information Resource, Baylor College of Medicine. To identify potential functional signatures in the *KALIG-1* protein, the sequence was used as a query to search the Prosite database<sup>67</sup>. The *KALIG-1* amino-acid sequence was also used as a query to search the non-redundant protein sequence database on the National Center for Biotechnology Information BLAST network service<sup>68</sup>. To characterize domains B and C better, subsequent searches of the non-redundant database used only the subregions of *KALIG-1* as a query. The highest scoring segment pairs resulting from this search were then aligned using the method of Smith and Smith<sup>64</sup>. Only one example each of closely related proteins was included in the alignment. The results of these alignments are shown in b. To identify other proteins that might contain the conserved domains B and C, subsequent searches of the non-redundant database used the consensus motif as a query.

### *KALIG-1* escapes X inactivation

Ubiquitous expression of *KALIG-1* is suggested by reverse transcription PCR (RT-PCR) experiments using RNA samples from several female fetal tissues, including brain, muscle, kidney, liver and intestine (Fig. 5a). Northern blot analysis gave no detectable hybridization bands, probably because of the low abundance of the *KALIG-1* transcripts. This precluded a quantitative analysis of *KALIG-1* expression in different tissues. Detection of *KALIG-1* expression by RT-PCR in cultured cell lines allowed us to test the X inactivation status of the gene. Reverse-transcribed RNA samples from four hybrid cell lines that retain either the active or the inactive X chromosome<sup>23</sup>

were amplified by PCR using oligonucleotide primers from the cDNA sequence. As shown in Fig. 5b, specific PCR products were observed in samples from both the active X and the inactive X hybrids indicating that this gene escapes X inactivation. Primers from the phosphoglycerate kinase gene (PGK1) and from the pseudoautosomal gene MIC2 which are genes subject to and escaping X inactivation, respectively, were used as controls for the amplification of the same cDNA samples and gave the expected results.

## Discussion

Pathways of neuronal migration are mediated by a variety of molecules influencing axon guidance, target recognition and cell-cell interactions<sup>24</sup>. An interesting model for such mechanisms is the joint migration of the neurons producing LH-RH and the terminalis and vomero-nasal nerves from the olfactory placode to the hypothalamus<sup>6</sup>. This migration pathway is interrupted in Kallmann's syndrome, indicating that a specific gene may be involved in the migration of this subset of neurons<sup>8</sup>. It has been suggested that the protein product of the *KAL* gene could be of the neural cell adhesion (N-CAM) type<sup>8</sup>.

We find that *KALIG-1*, is deleted, either partially or entirely, in all seven of our deletion and translocation patients, of whom four have intragenic breakpoints. Sequences highly homologous to this gene are present on the Y chromosome. These sequences have been mapped to Yq11.21 using a panel of Yq-deleted patients<sup>25</sup> (not shown). It is remarkable that within the region analysed we found the breakpoints of three Xp22.3/Yq11.2 translocations, two of which fall within different introns of *KALIG-1*. Sequence analysis of the region of the X and Y chromosomes corresponding to the t(X;Y)1 translocation breakpoint showed high similarity (95%) between X and Y-

derived sequences (data not shown). These data clearly indicate that X/Y translocation events in this region originated by abnormal pairing and crossing-over between homologous regions in Xp22.3 and Yq11.2, as previously suggested<sup>26</sup>.

The analysis of the predicted protein product of the *KALIG-1* gene has revealed the presence of three conserved domains: a four-disulphide-core domain<sup>20</sup>, domain A, described in many protease inhibitors and, with different spacing, in neurophysins<sup>27</sup>, and two domains (B and C) of about 30 amino acids each, both related to part of the fibronectin-like type III domain. Significant homology with domains B and C was found in some protein phosphatases and kinases, known to be involved in cell growth, and in many of the neural cell adhesion molecules which play an important part in axonal pathfinding by both cell-cell adhesion<sup>24</sup> and neurite outgrowth-promoting<sup>28</sup> mechanisms. The finding that domains B and C are also contained in some protein kinases and phosphatases is particularly intriguing as it has been suggested that both kinases and phosphatases might physically interact with extracellular matrix and cell adhesion molecules<sup>29,30</sup> during the modulation of neural development and morphogenesis. It is well established that LH-RH-producing neurons and vomero-nasal and terminalis nerves are physically associated in the developing brain, following a common migration pathway<sup>6</sup>, and that both types of neurons fail to migrate in Kallmann's syndrome<sup>8</sup>. The *KALIG-1* gene product might have a direct influence on axonal outgrowth, or alternatively might be involved in recognition mechanisms between these two types of neurons, allowing coordinated migration, though at present there is no experimental evidence to support either hypothesis. But it is clear from previous studies that the migration of this subset of neurons is a complex mechanism in which several molecules are involved. In this context, it is intriguing

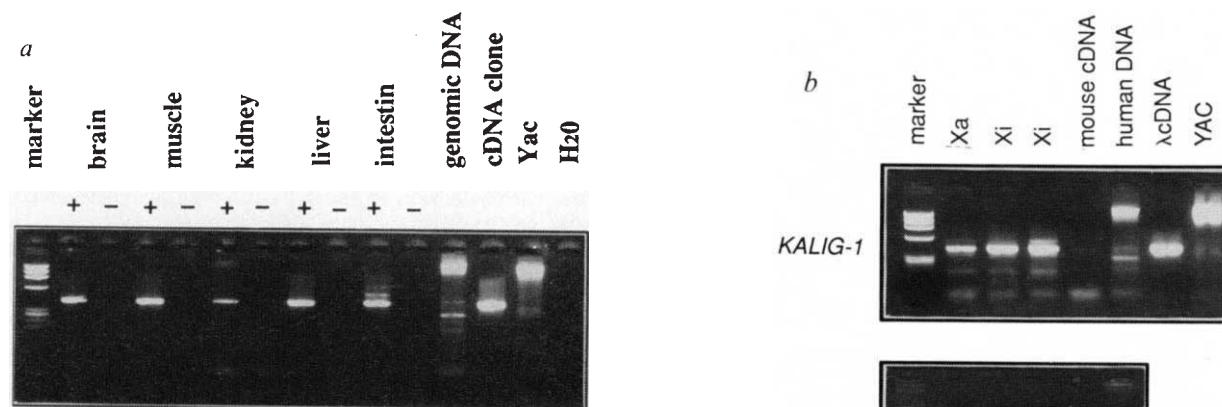


FIG. 5 Analysis of *KALIG-1* gene expression. *a*, Reverse transcription PCR DNA digested with of cDNAs from several tissues of an 18-weeks female fetus. Marker DNA was,  $\Phi$ X174 DNA digested with *Hae*III; cDNA clone represents *KALIG-1* cDNA clone; YAC, YAC X2; H<sub>2</sub>O, no DNA added. Plus and minus indicate presence and absence of reverse transcriptase (RT) in the RT reaction, respectively. As the primers are across a splice site with an intron of ~1 kb, they amplify two different bands in the cDNA (416 bp) and genomic DNA (~1.4 kb). *b*, X inactivation status of the *KALIG-1* gene. Oligonucleotide primers from the *KALIG-1* gene and from the human *PGK1* and *MIC2* genes were used for the amplification of human/mouse hybrids retaining the human active (Xa) or inactive (Xi) X chromosome hybrids. As the primers of the *KALIG-1* gene are across a splice site, spanning an intron of ~1 kb, they amplify two different size products in the cDNA (416 bp) and genomic DNA (~1.4 kb). Primers from the *MIC2* and *PGK1* genes amplify a product of 360 and 395 bp, respectively. The faint band observed in mouse cDNA lane using the *PGK1* primers is due to a nonspecific amplification product. Xa corresponds to cell line t60-12 (ref. 23). The two Xi cell lines are t48-1a-1Daz4a and t86-B1maz1b-3a (ref. 23);  $\lambda$ cDNA, *KALIG-1* cDNA clone; YAC, YAC X2; marker,  $\Phi$ X174 DNA digested with *Hae*III. METHODS. RT-PCR: total cellular RNA preparation and reverse transcriptase have been described<sup>58</sup>. The primers of the *KALIG-1* gene used were 569R

(5'-GGTGCTCCAAATTCAGGAAAAGCTG-3') and T312B (5'-CGGAGCTCCAC-CCTCTTCA-3') for both *a* and *b*. The positions of these primers shown in Fig. 2. Conditions for PCR were 40 cycles (94 °C for 1 min; 56 °C for 1 min; 72 °C for 3 min) with Cetus *Taq* polymerase and buffer. *MIC2* primers have been described<sup>69</sup>; *PGK1* primers were: PGK1r1 (5'-TCGGCTCCCTCG-TTGACCGA-3') and PGK1r2 (5'-AGCTGGGTGGCACAGGCTT-3'). PCR conditions for both *PGK1* and *MIC2* were 40 cycles (94 °C for 1 min; 53 °C for 1 min; 72 °C for 1 min).



to note that at least three genetic forms of Kallmann's syndrome, following different inheritance patterns, have been reported<sup>3,31</sup>, consistent with the involvement of different genes in the process.

Neurological abnormalities like synkinesia and nystagmus in patients with Kallmann's syndrome<sup>3-5</sup> might result from a lack of inhibitory fibres within the corpus callosum<sup>5</sup>, suggesting a defect in the migration of another subset of neurons; Kallmann's syndrome patients often suffer unilateral renal aplasia or hypoplasia, so the gene may also play a part in kidney development<sup>2</sup>. Alternatively, it is possible that these symptoms are caused by the involvement of contiguous genes. The incomplete penetrance of these features in families with Kallmann syndrome, however, makes deletion mapping efforts difficult.

We demonstrated that *KALIG-1* has a homologue on the long arm of the Y chromosome and escapes X inactivation. These peculiar features might reflect recent evolutionary changes in the region of the X chromosome in which this gene is located. The phylogenetic conservation of *KALIG-1* was studied by hybridizing cDNA probes to DNAs from male and female individuals belonging to different species (data not shown). Significant homology was detected in primate (chimpanzees, gorillas, macaques, cercopithecus and lemurs), bovine, rabbit and chicken DNA but not in hamster, mouse or *Drosophila melanogaster* DNA. Dosage analysis suggested X-linkage in chimpanzees, macaques and cercopithecus, but a pseudoautosomal (or autosomal) localization in lemurs, cows and rabbits.

Y-specific bands were observed in chimpanzees and macaques. Like *KALIG-1*, the steroid sulphatase gene (*STS*), which is located about 1.5 megabases distal to *KAL* locus<sup>14,18,19</sup>, has a homologue on the Y long arm<sup>32,33</sup>, escapes X inactivation<sup>34</sup> and is not conserved in the mouse<sup>33</sup>. It has been proposed that the region containing the *STS* and *KAL* loci was originally pseudoautosomal, as is the murine *STS* gene<sup>35</sup>, and was transferred to Yq11 by a pericentric inversion of the Y chromosome during evolution, followed by loss of function of the Y copies of the genes<sup>32,33</sup>. Although there is no definite proof that the Y copy of *KALIG-1* is inert, as is the human *STS* Y-specific pseudogene, preliminary data indicate that the Y chromosome copy is not expressed at least in hybrid cell lines. Escape from X inactivation may retain a functional significance for *KALIG-1* (or for *STS*), or it may represent the vestige of a pseudoautosomal location from a time when two copies of this gene were functional in males and females.

Kallmann syndrome may represent the first example of a human neuronal migration defect for which the gene involved has been identified. The *KALIG-1* gene provides a tool to study both sex chromosome evolution and mechanisms of neuronal migration in normal and pathologic conditions. Future studies will include the timing and tissue distribution of *KALIG-1* gene expression, the characterization of *KALIG-1* protein and of its action in cell migration, the isolation of *KALIG-1* homologues in other species and gene knock-out by homologous recombination for the creation of an animal model for Kallmann's syndrome.

**Note added in proof:** Since the manuscript was submitted we have found an intragenic deletion of the *KALIG-1* gene in a patient with isolated Kallmann syndrome. These data strongly support the hypothesis that *KALIG-1* is the Kallmann syndrome gene (D. Bick and A.B., in preparation). □

TABLE 1 Conservation of domains B and C

|                                                 | Ref. | B       | C       |
|-------------------------------------------------|------|---------|---------|
| <b>NEURAL CELL ADHESION MOLECULES</b>           |      |         |         |
| Axonal glycoprotein TAG-1 (TAG1)                | 28   | 3.0e-5  | 0.00098 |
| Neural cell adhesion molecule L1 (N-CAM-L1)     | 36   | 3.0e-5  | 0.0081  |
| Neural cell surface F3 (NCS-F3)                 | 37   | 3.0e-5  | 0.023   |
| Contactin                                       | 38   | 6.0e-5  | 0.023   |
| Integrin $\beta$ 4 subunit (ITB4)               | 39   | 0.028   | 0.0081  |
| Neuroglian (NRG)                                | 40   | 0.0018  | 0.066   |
| Neural cell adhesion protein (N-CAM-1)          | 41   | 0.11    | 0.023   |
| <b>PROTEIN KINASES</b>                          |      |         |         |
| Twitchin (Twitch)                               | 42   | 0.00024 | 0.066   |
| Myosin-light-chain kinase (MLCK)                | 43   | 0.00066 | >10     |
| Ros proto-oncogene tyrosine kinase (ROS)        | 44   | 0.31    | 0.0020  |
| Insulin receptor-related receptor ECK (ECK)     | 45   | 3.4     | 0.0081  |
| Hypothetical tyrosine kinase receptor eph (TKR) | 46   | 2.4     | 0.047   |
| Kinase-related protein sevenless (DVSVL5)       | 47   | 0.86    | 1.1     |
| <b>TYROSINE PHOSPHATASES</b>                    |      |         |         |
| Leukocyte antigen related protein (LAR)         | 48   | 0.00066 | 0.00024 |
| Protein-tyrosine-phosphatase delta (PTPD)       | 49   | 0.040   | 0.00024 |
| Protein tyrosine phosphatase DLAR (DLAR)        | 50   | 0.028   | 0.0040  |
| Protein tyrosine phosphatase DPTP (DPTP)        | 50   | 0.079   | 9.1     |
| <b>OTHERS</b>                                   |      |         |         |
| Fibronectin (FHNU)                              | 51   | 0.61    | 0.0020  |
| Adenylate cyclase                               | 52   | 0.11    | 0.023   |
| C-protein                                       | 53   | 0.20    | >10     |

Proteins that better fit the consensus of domains B or C. The expected number of matches with this level of similarity to a sequence in the database by chance alone is indicated. Abbreviations in parentheses are used in Fig. 4c.

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## LETTERS TO NATURE

## Images of aurorae on Jupiter from $H_3^+$ emission at 4 $\mu m$

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SINCE their discovery by Voyager<sup>1</sup>, aurorae on Jupiter have been regularly observed over the past ten years, from space at ultraviolet wavelengths<sup>2</sup> and from the Earth in the thermal infrared band<sup>3</sup> (8–13  $\mu m$ ). The ultraviolet emissions originate from H and H<sub>2</sub> in the highest part of the atmosphere, whereas the thermal infrared emissions originate from the stratosphere. Here we present images of near-infrared  $H_3^+$  emission, recorded simultaneously with high-resolution spectra. Having both imaging and spectral data provides us with a much improved opportunity to study the spatial and spectral characteristics of the  $H_3^+$  emission. We find that northern and southern emissions show strong spatial variation at the wavelengths of  $H_3^+$  emission. We suggest that the appearance of the jovian auroral regions at these wavelengths may be explained by a combination of localized  $H_3^+$  emission, reflection of sunlight by polar haze and stratospheric CH<sub>4</sub> emission in the 3.3–3.6  $\mu m$  range.

Following the successful spectroscopic detection of  $H_3^+$  lines in emission at 2  $\mu m$  and then 4  $\mu m$  in the auroral regions<sup>4–7</sup>, a new opportunity to study auroral activity by infrared imaging has been realized<sup>8</sup>.  $H_3^+$  ions are effectively generated in the auroral zone of Jupiter by ionization of H<sub>2</sub>, followed by reaction of H<sub>2</sub><sup>+</sup> with H or H<sub>2</sub>. The brightest  $H_3^+$  emissions are observed at 4  $\mu m$ , a spectral region in which Jupiter is dark because of strong absorption by jovian CH<sub>4</sub>.

The observations described here were part of a coordinated International Jupiter Watch effort, including the NASA Infrared Telescope Facility (IRTF), the Canada-France-Hawaii Telescope (CFHT) and the International Ultraviolet Explorer (IUE) satellite. The images were obtained at IRTF on 7 and 8 March, 1991 (UT), using the infrared camera ProtoCAM. Simultaneously, high-resolution spectra were obtained with the fourier-transform spectrometer (FTS) at CFHT, to monitor the spec-

tral characteristics and to determine rotational temperatures associated with the emission. Spectra covering the range 3.4–4.2  $\mu m$  were recorded with the FTS at a resolving power of 8,000, through a 2.5-arcsec aperture. The aperture was positioned on the planetary disk by offsetting from the galilean satellites, ensuring a pointing accuracy of 1 arcsec. A direct comparison of spectra and images is therefore possible. Here we concentrate on the spectral characteristics of the Protocam images by comparing the images with the FTS spectra. Interpretation of the morphology of the observed near-infrared emission is also important for stratospheric and ionospheric studies of Jupiter, in particular for investigating correlation of the emission with polar magnetic field lines, which govern particle precipitation in the auroral regions. First, however, it is necessary to understand the sources of all the 4- $\mu m$  emissions, including polar haze reflection and continuum, that are of non-auroral origins.

Figure 1 presents a series of six images of the southern hemisphere, corresponding to six wavelengths between 4.14 and 3.53  $\mu m$ , taken between 7:38 and 8:00 on 7 March (UT). The central meridian longitude (CML) is ~60° (hereafter all longitudes are in system III). They were taken almost simultaneously with the FTS spectrum presented in Fig. 2b, which is an average of five spectra recorded between 7:50 and 8:20, close to the brightest area seen in Fig. 1f. This spectrum shows prominent  $H_3^+$  emission lines.

To interpret structure in the images, it is necessary to separate the continuum from the spectral line emission. The continuum is mostly due to a combination of reflection of sunlight by tropospheric clouds and possible polar haze depending on wavelength. It is characterized in the images as a broad and symmetric feature outside the region of  $H_3^+$  emission. We denote this region the  $H_3^+$  'hot spot'. The six wavelengths, of which the positions are marked by circles or stars in Fig. 2a, were chosen to correspond to maxima (1b, d and f) and minima (1a, c and e) in  $H_3^+$  emissions. (See below for the distinction between circles and stars.) The contrast between continuum and hot-spot emissions can be seen by the difference between the two spectra in Fig. 2a: the solid line is a low-resolution spectrum obtained by smoothing the high-resolution spectrum in Fig. 2b with the bandpass of the circular variable filter (CVF) on the IRTF, and the dashed line is another spectrum, from which narrow  $H_3^+$  lines have been removed before smoothing. This convolution smoothes the narrow  $H_3^+$  emission peaks in Fig. 2b rendering widened bumps in Fig. 2a. The best contrast for  $H_3^+$  emission is at 3.99 and 3.53  $\mu m$  (images 1b, f), but the continuum still contributes 30% of the total flux at those wavelengths.

The intensity variation of the low-resolution spectra with wavelength is roughly comparable to individual ProtoCAM image intensities, as shown by the circles and stars in Fig. 2a.



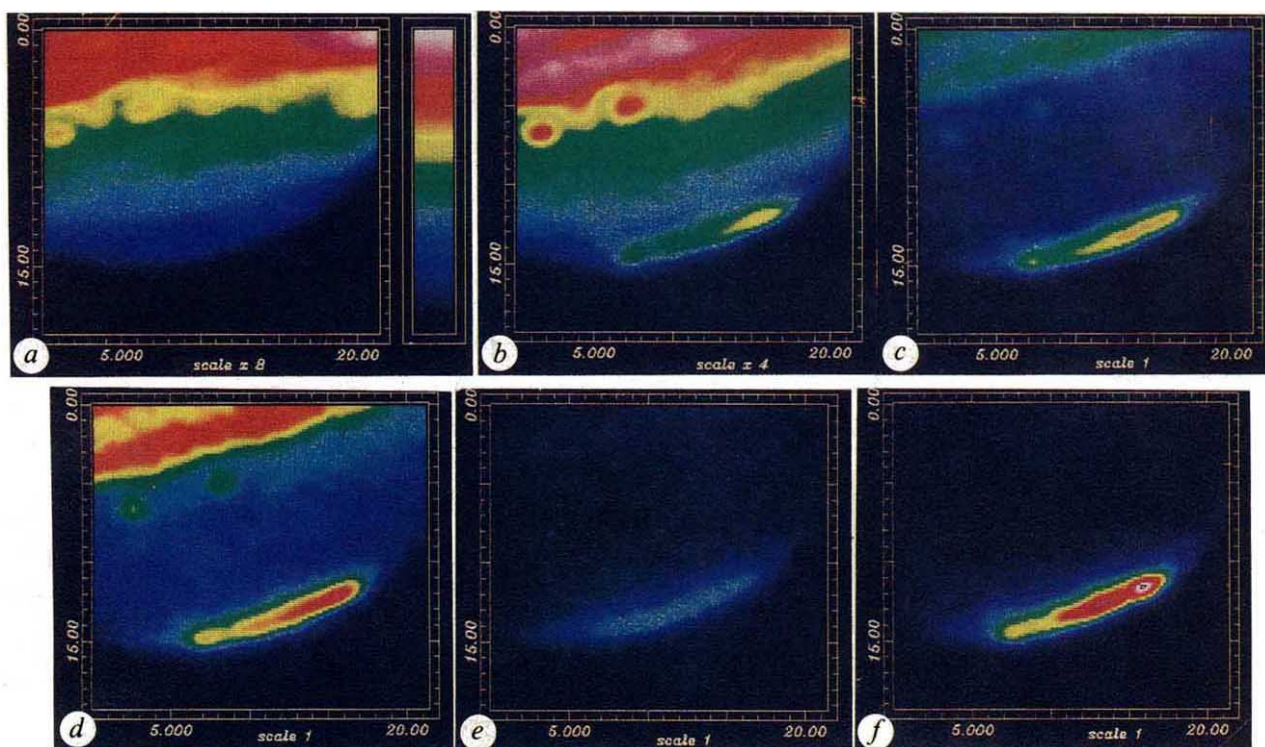


FIG. 1 South polar images at *a*, 4.14, *b*, 3.99, *c*, 3.85, *d*, 3.67, *e*, 3.58 and *f*, 3.53  $\mu\text{m}$ , recorded at 7:38, 7:42, 7:45, 7:51, 7:55 and 8:00 UT respectively, on 7 March 1991. CMLs are between 51 and 64°. The colour scale is linear for all images. The intensity scale is the same for all images except for *a* (divided by 1/8) and *b* (divided by 1/4). The *x*- and *y*-axes are in arcsec. Jupiter rotates from left to right, and north is up for all images in this paper. The position angle of Jupiter is  $\sim 16^\circ$ . The images presented here were obtained at IRTF using the ProtoCAM with an InSb array ( $62 \times 58$  pixels) through circular variable filters (CVFs) with a spectral resolving power of  $\sim 60$ . The pixel size was 0.35 arcsec, and the exposure time was 50 s. Calibration was made by comparison with the star HD772819, which was also used for frequent refocusing of the telescope. Using intensity profiles of the same star, the seeing was estimated to be  $\sim 0.70$  arcsec full width at half maximum. Data reduction includes sky subtraction and flattening, followed by smoothing with a triangular filter over three pixels.

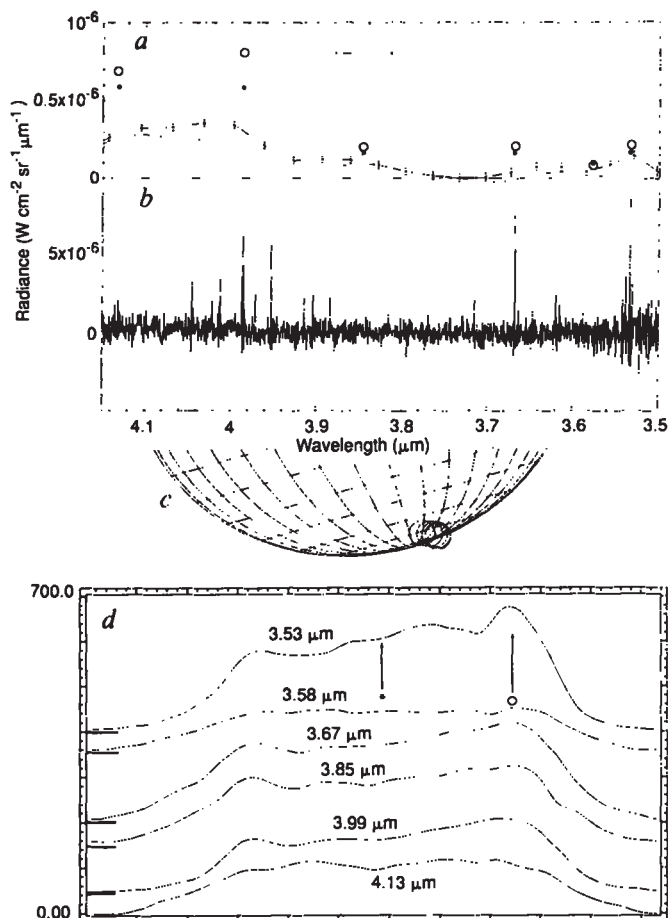


FIG. 2 Comparison of simultaneous spectra and images. *a*, solid line: FTS spectrum obtained by convolving the high-resolution spectrum in *b* with the CVF bandpass, which is roughly triangular. The horizontal bar is the CVF full width at half maximum. Dashed line: the same spectrum after the  $\text{H}_3^+$  lines have been removed, showing the pure continuum component. The circles and stars are calibrated ProtoCAM intensities measured for the bright hot spot and the central meridian in Fig. 1, respectively. Corresponding positions are marked in *d* as the right arrow and left arrow, respectively. *b*, Original high-resolution spectrum, with a resolution of  $0.00054 \mu\text{m}$ , recorded between 7:47 and 8:24 UT. The spectrum is an average of five short exposure spectra taken at the aperture positions of the five circles in *c*. CMLs are  $\sim 65^\circ$ . The FTS spectra were calibrated using  $\epsilon$  Leo (BS3873)<sup>12</sup>. *c*, The positions of the aperture of the spectrometer on the disk are shown as circles corresponding to a hot spot in Fig. 1*f*. Parts of the field of view ( $\sim 25\%$ ) are off the planet, causing some uncertainty in calibration. We consider this uncertainty to be a systematic error, which is still under detailed investigation. *d*, Limb-to-limb intensity profiles of the six images in Fig. 1, obtained along the  $63^\circ$  latitude and within the two dotted lines shown in *c*. The intensity scale is the same for all profiles except for those of 4.14 and  $3.99 \mu\text{m}$ , which are divided by 4. The profiles are shifted upward for clarity (except for the  $4.14 \mu\text{m}$  profile), and the base lines for the shifted profiles are marked.

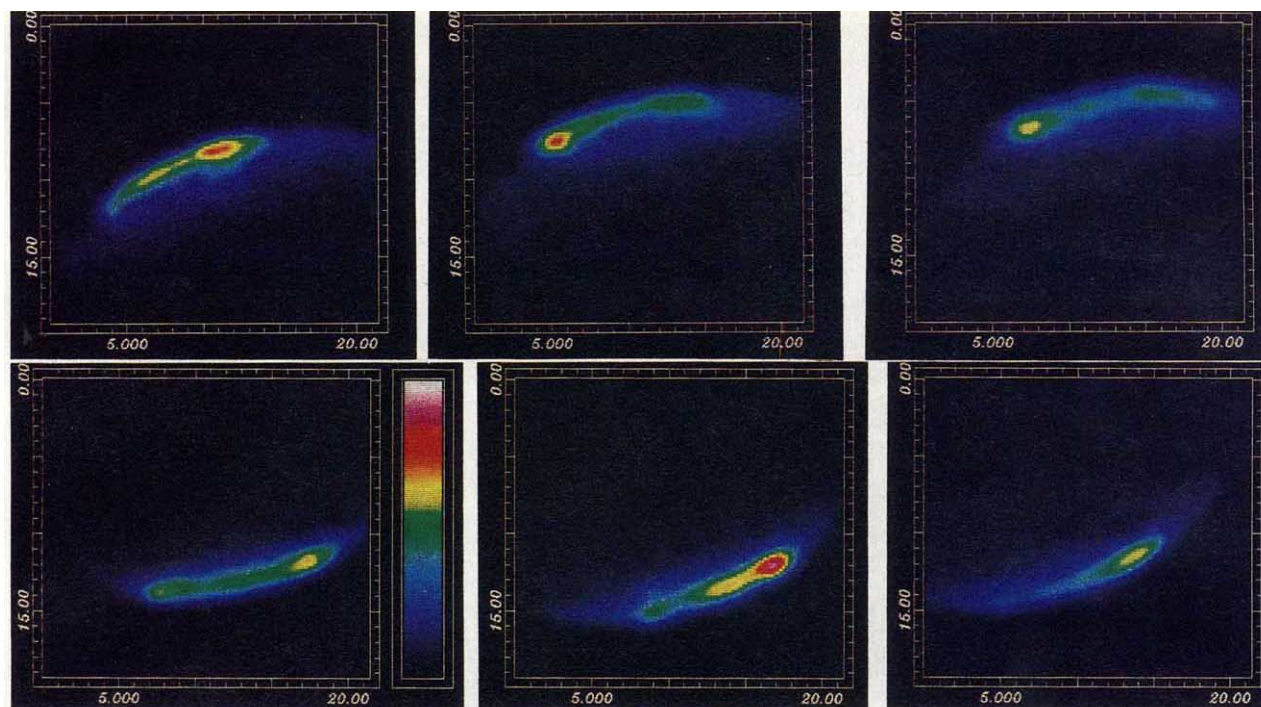


FIG. 3 Top row: a time sequence of three north polar images at  $3.53\ \mu\text{m}$ , where the continuum level is very low and only  $\text{H}_3^+$  lines occur. CMLs of the images are  $145^\circ$ ,  $185^\circ$  and  $205^\circ$ , and the corresponding times are 10:14, 11:20 and 11:56 on 7 March 1991 (UT), respectively. All the images have

the same intensity scale as the colour table. Bottom row: a time sequence of south polar images at  $3.53\ \mu\text{m}$ . CMLs are  $2^\circ$ ,  $91^\circ$  and  $167^\circ$ , and the times are 6:18, 8:44 and 10:50 on 7 March 1991 (UT).

There is discrepancy of a factor of  $\sim 2$  in absolute intensity, which comes from uncertainties in the calibration procedures and from different positions of measurement on the disk. The intensities of the circles and stars were respectively taken from the maximum brightness (right arrow in Fig. 2d) and from a central meridian position (left arrow in Fig. 2d). The negative fluxes of the dashed spectrum near  $3.7\ \mu\text{m}$  are within the error bars. Positions of the FTS aperture are plotted as circles in Fig. 2c.

Quantitative information can be obtained from the intensity profiles in Fig. 2d, which were obtained at  $-63^\circ (\pm 2^\circ)$  latitude for all six images in Fig. 1. The maximum brightness in the

profiles occurs at  $34^\circ (\pm 10^\circ)$  longitude. The profiles at  $3.68$  and  $3.85\ \mu\text{m}$  are mostly due to continuum emission, the morphology of which is similar to that of the polar haze previously observed<sup>9</sup> at  $2\ \mu\text{m}$ . The limb darkening is mainly caused by methane absorption above the haze and cloud layers, because of the longer line of sight through the atmosphere near the limb compared with the central regions. At  $3.53$  and  $3.99\ \mu\text{m}$ , where  $\text{H}_3^+$  emission is strong, considerable limb brightening is seen in the profiles, as expected from high-altitude, optically thin, emission lines. Finally, at  $4.14$  and  $3.99\ \mu\text{m}$ , cloud reflection of sunlight from the troposphere must be present in the continuum, because of low methane absorption at these wavelengths. Although there

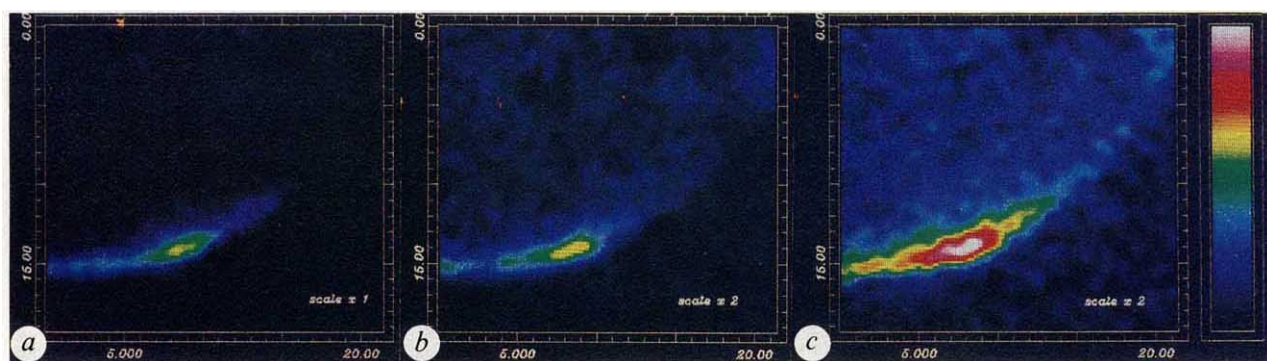


FIG. 4 ProtoCAM images at *a*,  $3.50\ \mu\text{m}$ , *b*,  $3.37\ \mu\text{m}$  and *c*,  $3.29\ \mu\text{m}$  taken at 11:33, 11:37 and 11:41 on 8 March, 1991 (UT), respectively. Relative intensity scales are indicated in the images, the fluxes were divided by a factor of 2 in *b* and *c*. Although we expect strong absorption by methane and very weak emission from  $\text{H}_3^+$  in these wavelengths, we see intense emission. Noise in *c* is high because of low atmospheric transmission at  $3.29\ \mu\text{m}$ .

The CMLs were  $\sim 273^\circ$  for the three images. The effects on  $\text{H}_3^+$  and possible methane line emission of differential Doppler shifts between the east and west limbs, because of jovian rotation, are minimal. This is because the telluric absorption, which is mainly due to methane lines, is weak at this wavelength, and the methane lines do not generally coincide with the  $\text{H}_3^+$  lines<sup>11</sup>.



are weak  $H_3^+$  lines around  $4.14\text{ }\mu\text{m}$ , the CVF bandpass completely smooths out the narrow lines. We therefore see a relatively dark pole, due to limb darkening, as shown in Fig. 1a. From the relative line intensities of  $H_3^+$  in the spectrum of Fig. 2b, a rotational temperature of  $950 \pm 100\text{ K}$  is derived. This is nominally lower than, but still consistent with, the temperature ( $1,100 \pm 100\text{ K}$ ) measured from  $2\text{-}\mu\text{m}$   $H_3^+$  emission in 1988.

In the first row of Fig. 3, we present a time sequence of three north polar images in the  $3.53\text{-}\mu\text{m}$  band, which is one of the most sensitive to  $H_3^+$  emission, showing variation of the  $H_3^+$  hot-spot feature as the planet rotates. In particular, the second image was taken at a CML of  $185^\circ$ , and shows no significant brightening at this longitude. A northern hot spot has been observed near  $180^\circ$  in the ultraviolet and at  $7.8\text{ }\mu\text{m}$  over the past 10 years (ref. 3), and it was present at the same longitude on 7 and 8 March (UT) in the ultraviolet, according to the IUE observations (R. Prange, personal communication). The second row of Fig. 3 presents a time sequence of southern hot-spot images. Intensity variations are clearly seen in this sequence. Limb brightening is observed when the  $H_3^+$  hot spot is close to the limb.

The three images in Fig. 4 were taken in a 10-min interval on 8 March, at wavelengths of  $3.50$ ,  $3.37$  and  $3.29\text{ }\mu\text{m}$ , near the centre of the  $\nu_3$  band of methane, where we expect strong absorption by methane but only weak emission from  $H_3^+$  (no FTS spectra were obtained at these wavelengths). In the images, however, there is a very bright area. The hot-spot intensity in the third image is  $\sim 10$  times as great as that of the rest of the planet. The absolute intensity at the emission peak of the third image in Fig. 4 is roughly the same as those of images in Fig. 3, which were taken on 7 March. All ProtoCAM images and FTS spectra, however, show a decrease in overall intensity of polar brightening by at least a factor of 3 on the second day. As we were not able to detect strong  $H_3^+$  emission lines in the  $3.4\text{--}4.2\text{ }\mu\text{m}$  range at that time, we do not expect that high- $J$  rotational  $H_3^+$  lines were strong near  $3.3\text{ }\mu\text{m}$ . We suggest that methane line emission from the upper stratosphere in the auroral zone, where temperatures are high, may be partly responsible for this bright emission. Reflection of infrared sunlight by the polar haze may also contribute, because the continuum morphology is similar to that of polar haze previously observed<sup>9</sup> at  $2\text{ }\mu\text{m}$ .

We constructed a methane emission model using existing models<sup>10,11</sup> and carried out a line-by-line calculation of radiative transfer, including methane absorption in the Earth's atmosphere and a jovian radial Doppler shift. We found that most of the emission of the  $3\text{-}\mu\text{m}$  band of methane originates from roughly the  $10\text{-}\mu\text{bar}$  level, where temperatures are  $300\text{--}400\text{ K}$ . The calculated intensity variation at the mean wavelengths of the images near the methane band is roughly consistent with the observed intensity variation, within a factor of two. Before we can unambiguously confirm or exclude the possibility of emission from the methane in the auroral zone, further FTS observations at high spectral resolution are needed.  $\square$

## Imaging Jupiter's aurorae from $H_3^+$ emissions in the $3\text{--}4\text{ }\mu\text{m}$ band

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SINCE  $H_3^+$  was first spectroscopically detected on Jupiter<sup>1,2</sup>, there has been considerable interest in using this simple molecular ion to probe conditions existing in the planet's auroral regions. Here we present a series of images of Jupiter recorded at wavelengths sensitive to emission by  $H_3^+$ , which reveal the spatial distribution of excited  $H_3^+$  molecular ions in the jovian ionosphere, as seen from Earth. We believe that they provide high-spatial-resolution images of polar aurorae on Jupiter. They suggest that the intensity of the auroral emission can vary on a timescale of an hour, a shorter period than had previously been noted. We also find that the spatial distribution of  $H_3^+$  emissions correlates only partially with the loci of auroral activity inferred from ultraviolet and longer-wavelength infrared observations. The  $H_3^+$  emission may therefore be controlled by auroral processes that are different from those responsible for the ultraviolet and infrared emissions.

The molecular ion  $H_3^+$  is produced in the jovian ionosphere at high latitudes by the ionization of  $H_2$  resulting from charged particle precipitation from the magnetosphere; the rapid reaction of  $H_2^+$  and  $H_2$  produces  $H_3^+$  (ref. 1). In subsequent reactions,  $H_3^+$  can provide both hydrogen atoms and protons, which can initiate complex hydrocarbon chemistry when produced below the homopause<sup>2</sup>, making it a sensitive probe of energy deposition from the magnetosphere into the atmosphere by high-energy particles.

Infrared emission from  $H_3^+$  was first detected on Jupiter in 1988 in the  $2\text{-}\mu\text{m}$  K window by the measurement of 23 lines of the  $2\nu_2(l=2) \rightarrow 0$  overtone band<sup>1,3</sup>, and identified from

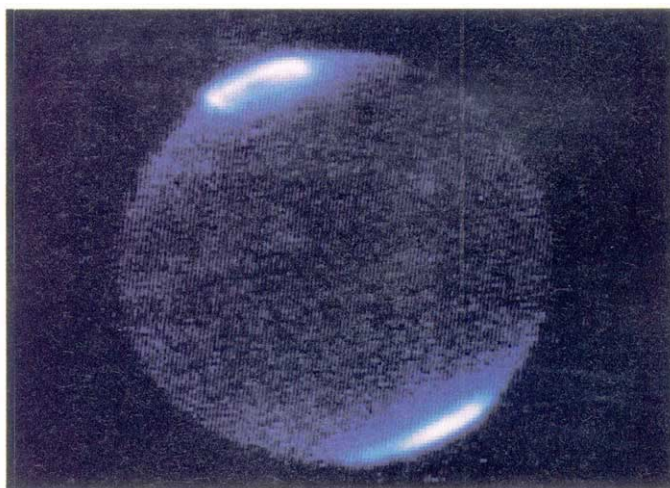


FIG. 1 Mosaic of nine ProtoCAM images of Jupiter measured at  $3.40\text{ }\mu\text{m}$  at  $\sim 1\%$  spectral resolution. Each frame had an effective integration time of 40 s. The detector in ProtoCAM is a  $58 \times 62$  InSb array. The equatorial diameter of Jupiter subtended  $45.7$  arcsec and the polar diameter  $42.7$  arcsec. The most intensive areas are shown as pink to white, and the least intense as dark blue to black. The System III CML of this image is  $163^\circ$ .

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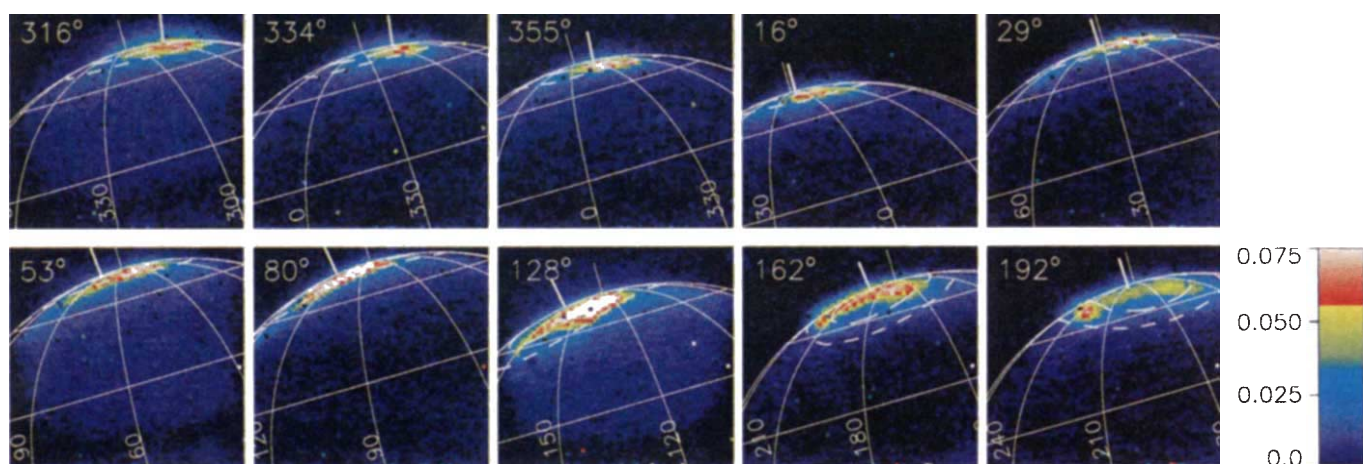


FIG. 2 Images of Jupiter's north polar region measured at  $3.55 \mu\text{m}$  on 24 February 1991. The CML for each image is indicated on the top-left corner. The planetary 'surface' fitted to each image is shown, corresponding to polar radius of 20.8 arcsec, equatorial radius of 22.25 arcsec, sub-Earth latitude of  $0.4^\circ$  and north pole position angle of  $16^\circ$ . Lines of latitude and longitude are shown at  $30^\circ$  intervals. The spin axis is indicated by a thin line, and the magnetic axis, offset from the spin axis of the planet by  $\sim 10^\circ$  towards

longitude  $202^\circ$  in the north, is indicated by a thick line. Also shown is the ultraviolet auroral oval (dashed lines) as determined from Voyager, as well as an estimated oval displaced by  $9^\circ$  towards the magnetic pole (dotted-dashed lines) to represent precipitation from near the magnetotail. The colour scale is linear, ranging from 0.0 to  $0.075 \text{ erg cm}^{-2} \text{ s}^{-1} \text{ sterad}^{-1}$ . Some images show saturation in this scale (the actual intensities can be as large as  $0.12 \text{ erg cm}^{-2} \text{ s}^{-1} \text{ sterad}^{-1}$ ).

calculated frequencies and Einstein  $A_{if}$  coefficients in conjunction with laboratory spectra<sup>4</sup>. Since then, the  $\text{H}_3^+$  fundamental  $v_2 \rightarrow 0$  spectrum has been measured in Jupiter, showing that the upper emitting levels are being populated thermally<sup>5</sup>. Other studies indicate that the temperature of the emitting gas is not constant in time and that there are variations in the relative strengths of the emissions in the northern and southern auroral zones<sup>6,7</sup>.

Baron and Owen<sup>8</sup> first obtained images of Jupiter at a wavelength sensitive to  $\text{H}_3^+$  in January 1991, using ProtoCAM, the infrared camera on the NASA 3-m Infrared Telescope Facility (IRTF). This camera incorporates three circular variable filters (CVFs) with spectral resolutions that are  $\sim 1\text{--}2\%$  of the bandpass (full width at half minimum). These three CVFs cover the wavelength range from 1.3 to  $5.5 \mu\text{m}$ . Here we present new images obtained on the nights of 1 and 22–24 February 1991,

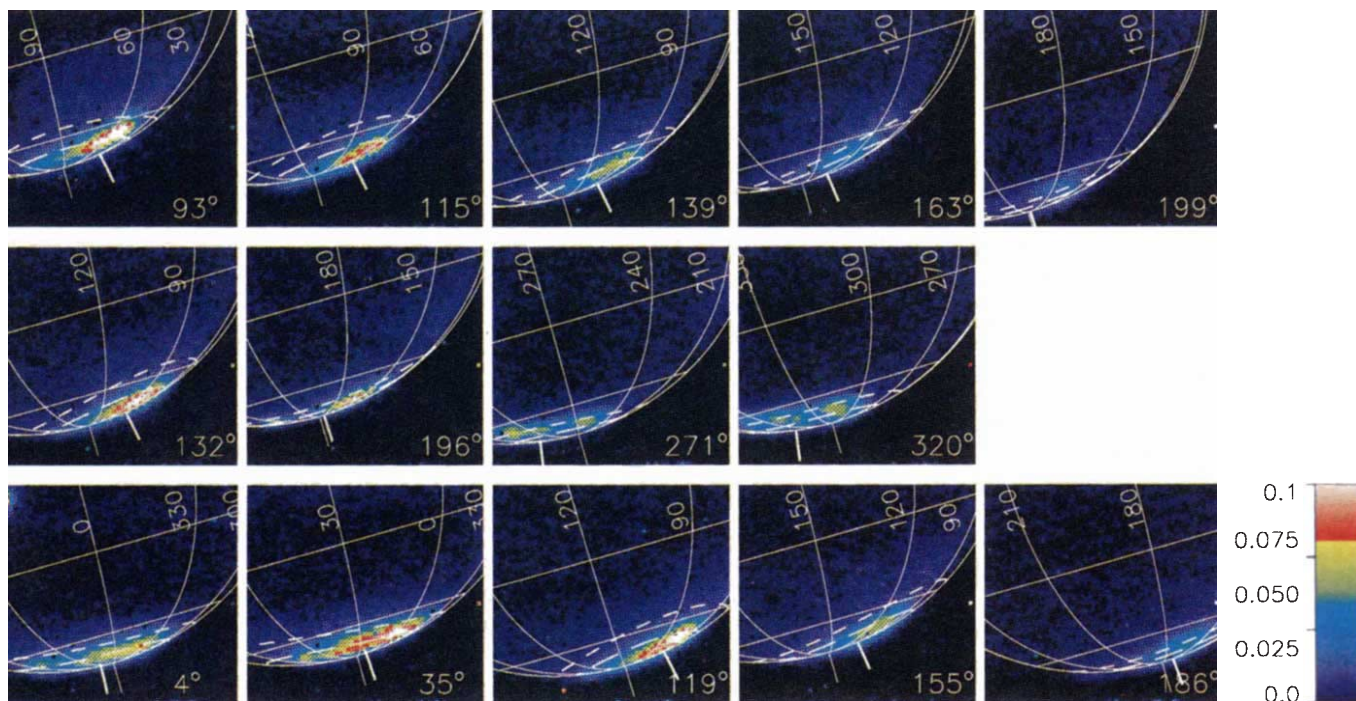


FIG. 3 Images of Jupiter's south polar region measured at  $3.4 \mu\text{m}$  for the nights of 22 February (top row), 23 February (middle row) and 24 February (bottom row). Theoretical auroral ovals mapped by the Io plasma torus

(dashed lines) and the magnetotail (dotted-dashed lines) are shown. The linear colour scale ranges from 0.0 to  $0.100 \text{ erg cm}^{-2} \text{ s}^{-1} \text{ sterad}^{-1}$ , and as in Fig. 2, some images show saturation in this scale.



Hawaii Standard Time. The image scale was 0.35 arcsec per pixel and the seeing on all nights except 24 February was <1 arcsec; on 24 February it was ~1.5 arcsec. The tracking during a single image acquisition was better than 0.1 arcsec. Although we made observations throughout the 1–5- $\mu$ m spectral range (S.M. *et al.*, manuscript in preparation), we are concentrating here on the interpretation of images obtained in the L window at 3.4 and 3.55  $\mu$ m. At the ~1% CVF resolution, these wavelengths correspond to strong  $H_3^+$  features formed by the merging of a number of R-branch transitions of  $v_2 \rightarrow 0$ . The P branch of  $v_3$  of methane also occurs in this wavelength range.

Figure 1 is a mosaic of nine reduced ProtoCAM frames (sky-subtracted, flattened and bad pixels removed) taken on 1 February, with the CVF centred on 3.4  $\mu$ m, to form a composite image of Jupiter. We attribute the overall darkness of the planet to the absorption of incident solar radiation by stratospheric methane. Bright emission features are evident at the poles of the planet, and we interpret these as being mainly due to  $H_3^+$  emission. To support this interpretation, we looked at various sequences of polar images at wavelengths throughout the L window. After compensation has been made for the effects of absorption by the Earth's atmosphere, the relative intensities of the peak polar emission correlate well with the expected  $H_3^+$  spectrum (S.M. *et al.*, manuscript in preparation). In contrast, the observed intensities do not follow the  $v_3$  spectrum of methane, although emission from this molecule around 7.8  $\mu$ m has been observed in the auroral zones<sup>9</sup>. The contribution of continuum around the poles was estimated from the flux we measured at 3.8  $\mu$ m (S.M. *et al.*, manuscript in preparation), which corresponds to a null in the  $H_3^+$  emission spectrum. The maximum emission at this wavelength was found to be ~0.025 erg cm<sup>-2</sup> s<sup>-1</sup> sterad<sup>-1</sup>, about 20% of the maximum northern emission at 3.55  $\mu$ m on 24 February, corresponding roughly to the boundary between dark and light blue on the saturated colour scale in Fig. 2. Emission above this level is therefore considered to be due to  $H_3^+$ .

Figure 2 shows a sequence of ProtoCAM frames taken at the jovian north pole at 3.55  $\mu$ m on 24 February 1991. Figure 3 shows similar sequences measured at 3.4  $\mu$ m in the southern hemisphere on 22, 23 and 24 February 1991. Central meridian longitudes (CML) in magnetic longitude system III are indicated. To aid interpretation, we have fitted the planet's oblate 'surface' at the time of the observation to each image to within  $\pm 0.5$  arcsec, using the low-level signal from the limbs in regions away from the  $H_3^+$  emission. This was accomplished by comparing the individual frames by eye with a full mosaic of the planet to which a calculated disk had been reliably fitted. The fitted mosaic shows slight limb darkening of the reflected continuum at low latitudes, which increases towards the poles. The  $H_3^+$  emissions are optically thin<sup>1</sup> and must show some limb brightening. Although extensive modelling would therefore be required to extract detailed quantitative information from the images, some general time-dependent and morphological characteristics can still be determined.

Comparison of the north polar images taken at CML 128° and 162° on 24 February indicates that the observed average emission between longitudes 135° and 155° and latitudes 65° and 75° weakened by ~25% during the hour between the images (Fig. 2). As this region has the same geometric aspect on both images, the differences in its intensity should not be due to limb brightening effects. Additionally, if we assume the morphology of the emission apparent in the image at 128° is maintained in the image at 162°, line-of-sight effects would cause the emission in the latter image to appear brighter, in contrast to what is observed. We therefore believe the most reasonable explanation of this effect is that it corresponds to an actual intensity variation of ~25% taking place in the jovian atmosphere during the hour between the two images. In the absence of detailed modelling, however, some contribution from geometric effects cannot be ruled out entirely. On the same night, strong emission at the

southern pole appears to grow between longitudes 330° and 60° in the hour between the images taken at CML 4° and 35° (Fig. 3, bottom row). Variation in emission strength among the three nights was also noted on both poles. For example, images taken on 22 February at CML 139° and on 23 February at 132° indicate that the southern emission was stronger on 23 February. Other data show that the north pole was also brightest on 23 February.

We have attempted to correlate the basic morphology of the  $H_3^+$ -emitting regions with that reported for the ultraviolet- $H_2$  and infrared-hydrocarbon aurorae, whose connection has not yet been determined. For the northern hemisphere (Fig. 2), we have plotted the loci of diffuse ultraviolet- $H_2$  auroral emissions as determined by the ultraviolet experiments on the Voyager missions<sup>10,11</sup> in 1979. It has been suggested<sup>12</sup> that this oval may be related to magnetospheric precipitation from the neighbourhood of the Io plasma torus. In addition, we have plotted an estimated oval displaced by 9° towards the magnetic pole to represent precipitation from regions further out towards the magnetotail. In the absence of Voyager data for the southern images we have plotted theoretical ultraviolet auroral ovals mapped by the torus and the magnetotail based on the O<sub>4</sub> plus current-sheet magnetic field model<sup>13,14</sup>. A peak in the northern ultraviolet emission at longitude 180° was detected by Voyager<sup>10,11</sup> and subsequent ultraviolet observations with the IUE satellite<sup>15</sup>, although recent IUE observations (of relatively poor spatial resolution) have also shown unusual longitudinal intensity distributions<sup>16</sup>. The south ultraviolet peak varies around longitude 20° (ref. 17). The infrared-hydrocarbon aurora<sup>9,18–21</sup> has been most extensively studied in its methane emission which consists of areas of high-level emission, possibly thermally excited, known as 'hot spots'. These occur at latitudes 60° in the northern hemisphere and ~70° in the south, and seem to be fixed in the north at longitude 180°, close to the ultraviolet peak, whereas their location in the south varies considerably in the region 300°–90° (refs 9, 18).

Our observations indicate that in both hemispheres, the regions of  $H_3^+$  emission are fixed with respect to the magnetic pole and are confined to latitudes above 60°, extending over the entire polar region. This extent of the emitting region is in agreement with recent detailed mapping of the 4.0- $\mu$ m spectrum of Jupiter (S. Ridgway *et al.*, manuscript in preparation). In the north (Fig. 2), the emissions show relatively bright spots centred around longitudes ~145° and ~230°. A local minimum occurs around longitude 180°. These features can be seen rising and setting in the sequence shown. Although we present results for only one night for the northern hemisphere, we observed similar morphology on the other two nights. The structure of the  $H_3^+$  emission region in the south is more complex (Fig. 3), due in part to the apparent temporal variations noted above. Taking the three nights together, strong emissions are seen from longitude 300° to 90°, with an additional weak maximum at ~260°.

We conclude that the observed  $H_3^+$  emission does not correspond exactly to any of the auroral ovals described above. This is particularly striking at 180° longitude in the north, where the peak of the ultraviolet auroral emission is usually observed, as is the methane hot spot. In the south, the overall longitudinal region of the methane emission as well as that of the ultraviolet emission seems to coincide with that of the  $H_3^+$  emission, but more detailed, time-resolved comparisons are required.

Further studies of the jovian ionosphere in the auroral regions are required to provide mechanisms to explain the basic results presented here. The possibility that the  $H_3^+$  emissions are controlled to some extent by auroral processes<sup>12</sup> different from those responsible for the ultraviolet and infrared-hydrocarbon emissions has to be explored. But a proper assessment of the relation of the various types of auroral emissions can only be made after detailed modelling of the dependence of the emissions on variables that may vary spatially, such as the atmospheric level at which the incoming magnetospheric particles deposit energy<sup>15</sup>, and that may affect the different types

of emissions in different ways. Nevertheless, our preliminary results clearly demonstrate that infrared spectral imaging provides a powerful tool for jovian auroral studies, permitting measurements of the spatial extent and temporal variations of auroral activity on Jupiter at sub-arcsec angular resolution. Concurrent data on  $\text{H}_3^+$  and methane as well as  $\text{H}_2$  emissions would be extremely helpful, as well as observations to determine just how rapidly the variations noted in  $\text{H}_3^+$  emission strength occur. Such data, combined with other measurements, will provide a strong constraint on physical, chemical and dynamical models of the jovian system. □

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## Local oxygen ordering in superconducting $\text{YBa}_2\text{Cu}_3\text{O}_{6.4}$ observed by neutron diffraction

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THE superconducting transition temperature,  $T_c$ , in the  $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$  system is known to depend not only on the oxygen stoichiometry<sup>1</sup>, but also on the degree of oxygen order<sup>2,3</sup>. Observations of oxygen ordering structures by electron microscopy<sup>4,5</sup> have led to the suggestion that the '60-K plateau' near  $x=0.5$  in the curve of  $T_c$  versus  $x$  arises from an ordered structure (the 'ortho-II phase') in which Cu–O and Cu chains alternate in the basal plane<sup>6</sup>. Until now, however, the ortho-II phase has been seen only by electron diffraction, except for one observation at an oxygen stoichiometry of  $x=6.7$  by X-ray diffraction<sup>7</sup>. Compared with true bulk methods such as X-ray and neutron diffraction, electron diffraction suffers from the disadvantage that only a very small portion of the sample is probed. Here we report the observation, by neutron diffraction, of local ortho-II ordering in an  $\text{YBa}_2\text{Cu}_3\text{O}_{6.4}$  crystal ( $T_c=38$  K). The observed correlation lengths along the principal axes are  $24b$  (along the chains),  $10a$  and  $2c$ . Our observation of the existence of the ortho-II phase for an oxygen stoichiometry close to the minimum for which superconduc-

tivity is observed ( $x \approx 0.4$ ) supports existing theoretical models<sup>8,9</sup>, in particular one<sup>8</sup> that relates  $T_c$  to the presence of two types of ordered domain.

In  $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$  superconductivity occurs for  $x \geq 0.4$ , and  $T_c$  increases with increasing oxygen content to 93 K at  $x=1$ . The curve has two plateaux, near  $x=1$  ( $T_c=90$ ) and  $x=0.5$  ( $T_c=60$  K)<sup>1</sup>. The importance of local oxygen ordering for superconductivity in  $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$  was demonstrated in refs 2 and 3, where the increase of  $T_c$  as samples were annealed at room temperature was attributed to progressive local ordering of oxygen atoms in the basal plane.

Poulsen *et al.*<sup>8</sup> studied the oxygen ordering in the basal Cu–O planes by computer simulations and its influence on  $T_c$  through a simple charge-transfer process. Assuming that only the ordered ortho-I and ortho-II phases (ortho-I: the normal orthorhombic phase for  $x=1$ ; ortho-II: a phase near the composition  $x=0.5$ , consisting of alternating full and empty Cu–O chains) contribute to superconductivity, and that the ordered domains have to be at least a certain size, they could obtain a theoretical prediction of  $T_c(x)$  in close quantitative agreement with the two plateaux found experimentally. Their model description of  $T_c$  as a function of  $x$  is based on the two-dimensional model of anisotropic next-nearest-neighbour interaction (the ASYNNNI model) first introduced by de Fontaine *et al.*<sup>9</sup>. Despite its simplicity, the ASYNNNI model can account in detail<sup>10,11</sup> for many of the most significant experimental results, including the stability of the ortho-II phase, the structural phase boundary between tetragonal disordered and orthorhombic ordered structures<sup>12</sup>, and thermodynamic anomalies observed in the oxygen equilibrium pressures<sup>12,13</sup>. But there are experimental observations for which the ASYNNNI model cannot account, such as the ortho-III phase<sup>4</sup>, which requires longer-range interactions<sup>14</sup> than were included in the original ASYNNNI model. Another significant result that cannot be accounted for is the superstructure formation in the tetragonal disordered phase, which has been established recently<sup>15</sup> from single-crystal neutron-diffraction studies of a sample with oxygen stoichiometry  $x=0.35$ . From the intensities of superstructure reflections a tetragonal oxygen ordering model ( $2\sqrt{2}a \times 2\sqrt{2}a \times c$ ) was derived. In this structure, 'half-filled' Cu–O chains alternate with 'quarter-filled' chains.

The importance of oxygen ordering for superconductivity in  $\text{YBa}_2\text{Cu}_3\text{O}_{6+x}$  calls for an increasing knowledge about the oxygen ordered structures in all parts of the phase diagram. Until recently, only the detailed structures of the endmember compounds,  $x=0$  and  $x=1$ , could be determined by neutron and

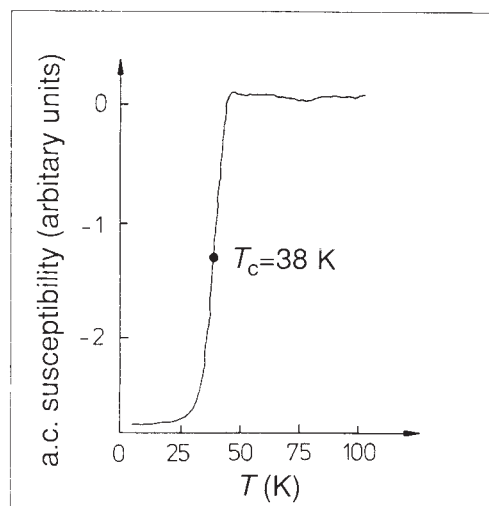


FIG. 1 The a.c. susceptibility curve giving  $T_c=38$  K with a transition width of 5 K.



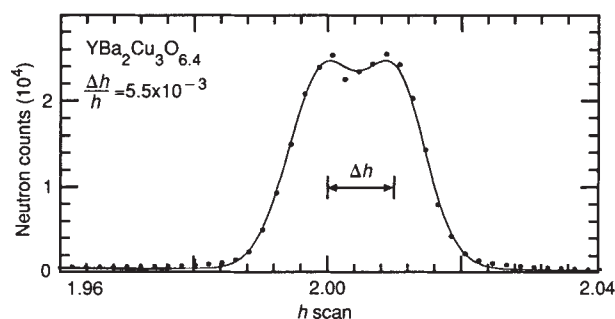


FIG. 2 The twin reflections (200) and (020) from  $\text{YBa}_2\text{Cu}_3\text{O}_{6.4}$  showing an orthorhombicity  $(b-a)/b=0.0055$ .

X-ray diffraction. For intermediate oxygen concentrations, defect ordering structures had been observed by electron diffraction<sup>4,5</sup>, but until recently there had been very few observations by X-ray and neutron diffraction, probably because the predominantly two-dimensional character of the ordering can only be studied by these techniques if sufficiently large and well equilibrated single-crystal samples are available. For the ortho-II phase,  $x=0.5$ , there is restricted information from neutron powder work on  $\text{YBa}_2\text{Cu}_3\text{O}_{6.6}$ , two small peaks were detected and attributed to ortho-II domains with a large uncertainty in the correlation length of  $26(\pm 24)$  Å. The X-ray work on a single crystal of  $\text{YBa}_2\text{Cu}_3\text{O}_{6.7}$  indicates ortho-II domains with correlation lengths of 21, 16 and 9 Å in the  $a$ ,  $b$  and  $c$  directions.

We grew a single crystal of composition  $\text{YBa}_2\text{Cu}_3\text{O}_{6.7}$ , measuring  $6 \times 3 \times 2$  mm, from CuO-BaO flux in an alumina crucible by the slow-cooling method<sup>17</sup>. After annealing in oxygen, the crystal showed a  $T_c$  of 87 K. To develop the ortho-II phase, the crystal was reduced from an oxygen concentration near  $x=1.0$  to  $x=0.4$  by stepwise cooling from 650 °C to 150 °C in 200 h under controlled oxygen equilibrium pressures given by Specht *et al.*<sup>18</sup>. A closed-volume system with sufficiently low oxygen content to prevent further oxidation was used below 350 °C. At 150 °C the sample was subjected to a long-time (400 h) ageing to establish oxygen ordering. After this treatment, the  $T_c$  determined by a.c. susceptibility measurements (Fig. 1) was 38 K, with a transition width of 5 K.

Neutron measurements were performed on the triple axis spectrometer TAS 1 at Risø National Laboratory (RNL). Special care was taken to suppress the higher harmonics of the

wavelength used,  $\lambda = 4$  Å. We inserted beryllium filters between the graphite monochromator and the sample, and between sample and the graphite analyser. To improve the signal-to-noise ratio and to separate inelastic scattering events, we operated the spectrometer in the elastic mode.

Superstructure reflections ( $hkl$ ) were observed with  $h = (2n+1)/2$  and  $k, l, n$  integers. The ortho-II-phase exists only in domains of limited size because the reflections are significantly broadened or diffuse. The crystal is twinned with equal amounts of different domains with interchanged  $a$  and  $b$  axes. The superstructure reflections of the two domains do not overlap. We used the TAS 7 triple axis spectrometer at RNL with neutrons of wavelength  $\lambda = 2.97$  Å to determine the orthorhombicity  $(b-a)/b = 0.0055(12)$  from the close-lying (200) and (020) reflections resulting from different domains (Fig. 2), and to determine the length of the  $c$ -axis:  $c = 11.73(2)$  Å.

To avoid using destructive methods giving direct information on the oxygen concentration of the sample, we determined  $x$  indirectly by comparing our experimental values of  $T_c$ , orthorhombicity and the length of the  $c$ -axis with literature values of their  $x$ -dependence. Although these values may vary significantly with the degree of oxygen order<sup>3</sup>, and thereby with the sample preparation technique, we found relatively consistent results from comparison with data obtained under equilibrium conditions<sup>12</sup>, by quenching from 520 °C (ref. 19) and by zirconium-gettered annealing at 440 °C (ref. 20). The combined results give from  $T_c$  values:  $x = 0.38(2)$ , from orthorhombicity:  $x = 0.38(3)$  and from the  $c$ -axis:  $x = 0.48(10)$ . In consequence, we estimate  $x = 0.40(5)$  to be a likely oxygen stoichiometry for the sample.

The profiles of the  $(\frac{1}{2}00)$  superstructure reflection along different directions of the reciprocal space are shown in Fig. 3. In all directions, the width of the diffuse superstructure reflection is much broader than the instrumental resolution, which was determined from scans through the (100) and the (001) ( $l=1, 2$  and 3) reflections. The sizes of the domains with ortho-II structure may be derived assuming a gaussian distribution of domain size from the diffuse peak width of gaussian shape,  $\xi = 1/\Delta_{\text{FWHM}}$ . The results show a strong anisotropy in the correlation length of the ortho-II-phase in  $\text{YBa}_2\text{Cu}_3\text{O}_{6.4}$  (inset of Fig. 3). The tendency of the system to build Cu-O chains in the basal plane is reflected by a correlation length  $\xi_b = 24b$  (90 Å) in chain direction which is more than twice as large as the in-plane correlation perpendicular to the chains,  $\xi_a = 10a$  (40 Å). In the  $c$ -direction there is only a correlation between nearest and next-nearest basal-planes,  $\xi_c = 2c$  (22 Å), indicating that the ordering is predominately two-dimensional.

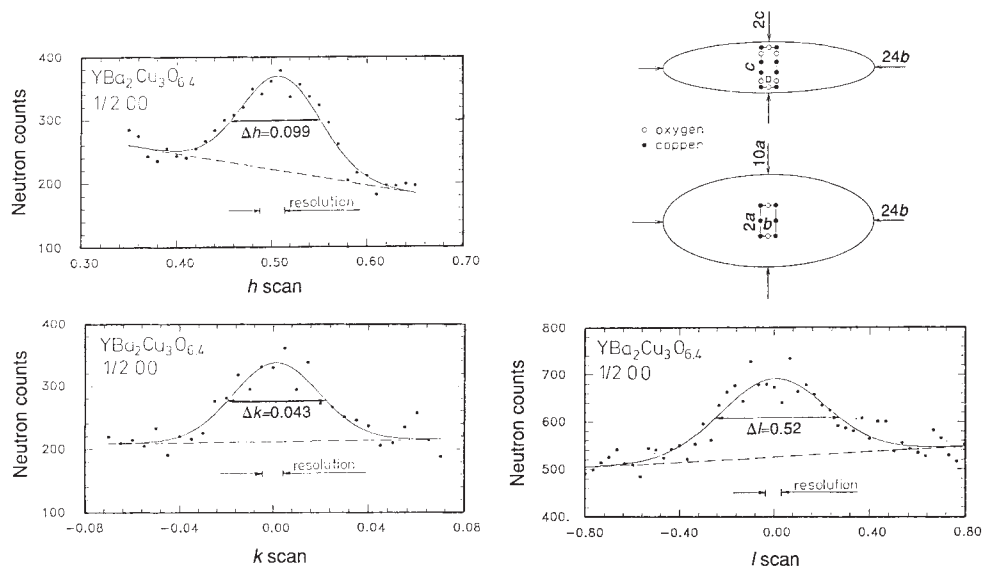


FIG. 3 Profiles of the  $(\frac{1}{2}00)$  superstructure reflection along the main axes of reciprocal space and corresponding average dimensions of the ortho-II domains in real space.

By scaling the integrated intensity of the superstructure reflection with that from a Bragg reflection we estimate that  $\sim 60\%$  of the crystal volume has the ortho-II structure. This is interesting in relation to the minimal model presented by Poulsen *et al.*<sup>8</sup>, which correlates  $T_c$  to the percentage of oxygen ordered sites in minimal size domains. For ortho-II the minimal size of domain is  $8 \times 8$  oxygen sites, which is smaller than but of the same order of magnitude as the average domain size determined from our diffraction data. Thus the 60% oxygen that we find in ortho-II phase domains should give  $T_c = 0.6 \times 60 \text{ K} = 36 \text{ K}$ , in striking agreement with the experimental observation of 38 K.

By changing the wavelength to  $\lambda = 2.4 \text{ \AA}$  we observed further superstructure reflections at  $(\frac{1}{2} 1 0)$ ,  $(\frac{1}{2} 2 0)$ ,  $(\frac{1}{2} 0 1)$  and  $(\frac{3}{2} 0 2)$ . From these data we cannot give a detailed description of the defect ordering structure including possible cation relaxations, but the results have established the stability of the ortho-II phase domains for oxygen stoichiometries just exceeding the critical value for superconductivity. This is in agreement with the ASYNUNI model, which predicts the ortho-II phase to be stable in an oxygen concentration range from nearly  $x = 0.3$  to  $x \approx 0.7$ , but our data do not indicate a long-range ortho-II phase. Previous attempts to detect the ortho-II phase by bulk diffraction techniques (X-rays or neutrons)<sup>7,16</sup> have succeeded only at  $x \geq 0.5$ . Our observation of this phase at  $x = 0.4$  support the idea that it is the ortho-II structure, connected with  $T_c = 60 \text{ K}$ , that accounts for the extension of the 60-K plateau down to nearly  $x = 0.4$ .

For further quantitative comparisons with model calculations,

it is of interest to establish how the size of the ortho-II domains and the ordered fraction change as the oxygen concentration varies across the 60-K plateau. In this respect it is also important to establish the sizes and fractions of other types of domains such as the ortho-III across the whole oxygen concentration range. The scarcity of single crystals of sufficient size and quality, and the time required for oxidizing and equilibrating large single crystals and collecting data from low-intensity diffuse scattering, mean that these studies will take some time to complete.  $\square$

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## Absence of weak-link behaviour in $\text{YBa}_2\text{Cu}_3\text{O}_7$ grains connected by $90^\circ$ [010] twist boundaries

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ONE of the obstacles to developing applications of the high- $T_c$  copper oxide superconductors is the low critical current density ( $J_c$ ) of the bulk polycrystalline materials, due to the presence of grain boundaries. On the other hand, these same grain boundaries can be put to good use in device applications, where their low  $J_c$  allows them to be used as 'weak links' in Josephson junctions<sup>1,2</sup>. Dimos *et al.*<sup>3,4</sup> have shown that the transport properties of certain twist and tilt boundaries degrade as the misorientation angle increases, suggesting that high-angle grain boundaries are generally deleterious to the overall  $J_c$ . More recently, however, Babcock *et al.*<sup>5</sup> reported weak-link-free behaviour at high magnetic field in an  $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$  (YBCO) bicrystal containing two kinds of  $90^\circ$  boundary; they were not able to distinguish between the characteristics of the two types, which were electrically in parallel. Here we report the observation of  $90^\circ$  [010] twist boundaries in {103}-oriented YBCO thin films. (Because of the twin microstructure, we do not distinguish between the  $a$  and  $b$  axes.) Across these boundaries the  $J_c$  and normal-state conductivity are as high as those of high-quality  $c$ -axis-oriented films with no high-angle grain boundaries.

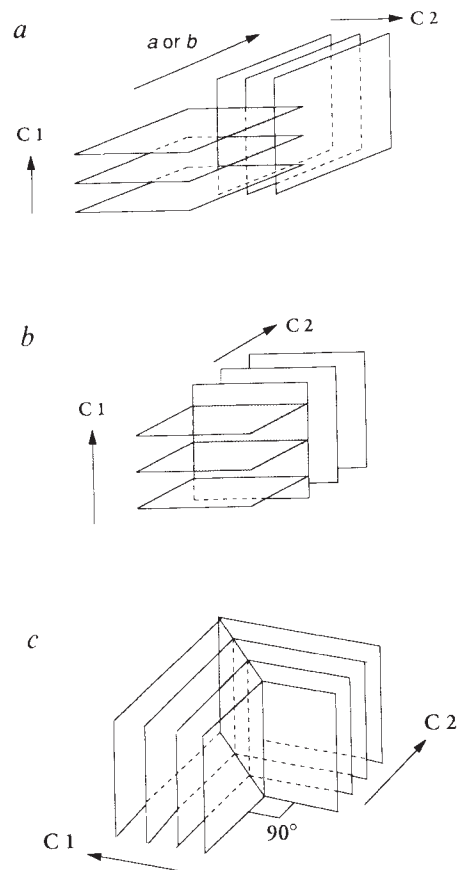
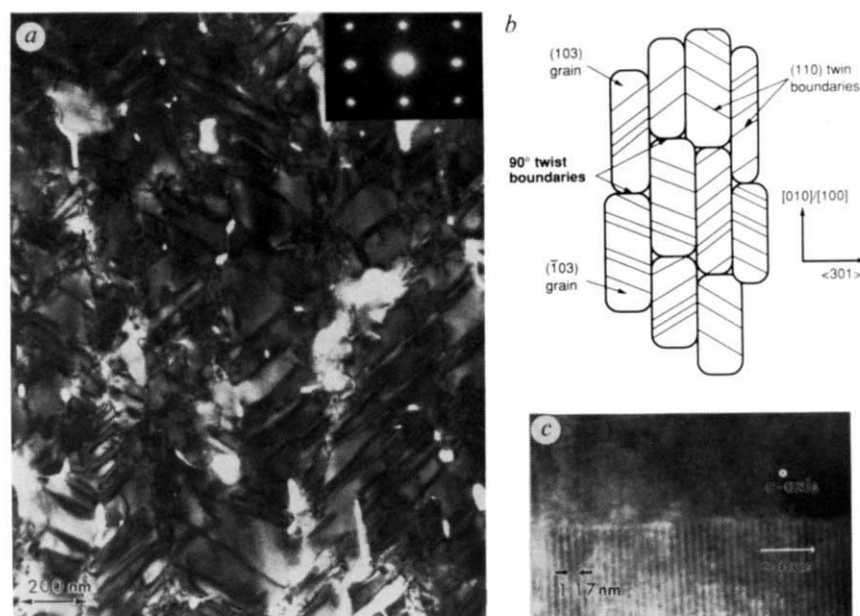


FIG. 1 Schematic diagrams of various  $90^\circ$  grain boundaries; a, a  $90^\circ$  [010] twist boundary (type A); b, a  $90^\circ$  [010] basal-plane-faced tilt boundary (type B); and c, a  $90^\circ$  [010] symmetrical tilt boundary (type C).



FIG. 2 *a*, Planar transmission electron micrograph and *b*, an idealized schematic of the grain and twin boundary structure of a {103} YBCO thin film. The electron diffraction pattern in the inset confirms the in-plane texture and absence of (110) grains. *c*, The 90° [010] twist boundary in which the sample is rotated 45° about the [010] direction. The *c*-axis fringes of the bottom grain can be seen. *d*, Three-dimensional schematic diagram of (103)/(103) YBCO films showing a 90° [010] twist boundary. The twins are not shown here.



grew 4,000-Å *c*-axis films on (100) MgO and (100) SrTiO<sub>3</sub> substrates to compare properties. The substrate block temperature was held at 720 °C.

The film textures were investigated by X-ray diffraction using a four-circle diffractometer with a Cu Kα source. From the  $\theta$ -2 $\theta$  scan of a YBCO film grown on (101) LaAlO<sub>3</sub>, we observed only peaks due to YBCO from the {103} and {013} orientations. In a similar scan for a film grown on a (101) SrTiO<sub>3</sub> substrate, we could not identify a {103} peak because of overlap by the strong (101) substrate peak. The in-plane texture and absence of (110) oriented grains were also investigated by off-axis  $\Phi$  scans of the {102} peaks. It was confirmed that both possible variants of the {103} orientation are present. The first orientation is a (103) grain: substrate [010] parallel to (//) YBCO[010] and substrate [101]//YBCO[301]. The second orientation is a (103) grain: substrate [010]//YBCO[010] and substrate [101]//YBCO[301]. The orientation of the CuO<sub>2</sub> planes changes by 90° between these two variants.

Nucleation and growth of both orientations on the substrate defines the grain and grain-boundary structure of the film, as determined by plan-view transmission electron microscopy (TEM). Figure 2 shows the TEM image and accompanying schematic of the grain structure. The grains nucleate in the tetragonal phase regime in either the (103) or (103) orientation. They are elongated in the [010] direction, which is the fast growth direction; the perpendicular <301> direction is an ordering direction and therefore a slow growth direction. The tetragonal-to-orthorhombic transformation twins, with (110) boundaries oblique to the film plane, form within these grains during cooling. This gives the additional {013} peak in the X-ray scan. Note that all the grains are not perfectly aligned along the [010] direction. As we indicate in Fig. 2, the predominant boundaries in the [010] direction are 90° [010] twist boundaries.

Figure 2*d* is a three-dimensional schematic diagram of a {103} YBCO film; note that the CuO<sub>2</sub> planes are at either -45° or +45° to the substrate surface depending on whether the orientation is (103) or (103). In both of the main crystallographic directions, therefore, there are 90° boundaries; the orientation of the CuO<sub>2</sub> planes changes by 90° across the boundary. Twins within the grain will cause the [010] and [100] directions to alternate, but will not change the orientation of the superconducting planes. In the <301> or <031> direction that shows inferior transport properties, we find the types of boundaries shown in Fig. 1*b* and *c*; these will be discussed elsewhere

The work of Dimos *et al.*<sup>3,4</sup> was carried out on single grain boundaries in YBCO films deposited on SrTiO<sub>3</sub> bicrystals. The misorientation angle between two crystals was varied from 0 to 45°, and  $J_c$  decreased by two orders of magnitude when the misorientation angle increased beyond 10°. It is not possible to make 90° grain boundaries using this technique, because the substrate is cubic.

In the bicrystal of Babcock *et al.*<sup>5</sup>, the two types of 90° boundary (Fig. 1) are a [010] twist boundary (type A) and an [010] basal-plane-faced tilt boundary (type B). Their conclusion of weak-link-free behaviour was based on the fact that the  $J_c$  across both boundaries in parallel,  $\sim 3 \times 10^3$  A cm<sup>-2</sup> at 73 K, was not lower than the intra-grain  $J_c$ , and on the features of the characteristic curves of  $J_c$  against magnetic field and current against voltage. For technological purposes, however, it is desirable to measure weak-link behaviour in the range 10<sup>4</sup>-10<sup>6</sup> A cm<sup>-2</sup>. Chan *et al.*<sup>10</sup> have reported that some 90° misoriented grain boundaries do not reduce  $J_c$ .

The measurements reported here were made across boundaries of type A, found in the [010] direction of {103}-oriented films (that is, films with the {103} crystallographic planes parallel to the film surface).

Films of thickness 4,000 Å were grown *in situ* by a 90° off-axis sputtering technique<sup>6,7</sup>. Both (101) SrTiO<sub>3</sub> and (101) LaAlO<sub>3</sub> substrates have been used. We define the substrates as having a (101) orientation instead of (110) for clarity. The directions of the substrate edges are [010] and [101]. We simultaneously

(C.B.E. *et al.*, manuscript in preparation).

In the [010] or [100] direction, the grain boundaries are all of a single type: 90° twist boundaries about the [010] or [100] axis. This is the boundary shown in Fig. 1a. As we cannot distinguish between the [100] and [010] directions (and for simplicity), we refer to these boundaries as 90° [010] twist boundaries; we note that in addition to their twist character some of these boundaries may also have a twin component where [010] changes to [100]. The orientation relationship across these boundaries was confirmed by imaging and micro-diffraction when the TEM sample was tilted 45° about the [010]/[100] axis. In this orientation the *c*-axis lattice fringes are visible in one variant but not the other, as shown in Fig. 2c.

There are two other types of boundaries in the [010]/[100] direction. One is the pure (110) twin boundary across which the [010] and [100] directions alternate. These boundaries occur in all YBCO material, including *c*-axis films; it has been shown that they do not act as weak links. The other type of boundary is an antiphase boundary across which there is a 1/3 shift of the CuO<sub>2</sub> planes; these occur mainly because of out-of-registry nucleation of adjacent grains in the same direction. Although these boundaries have not been isolated and characterized, there is no previous evidence suggesting that they are weak-link boundaries (they occur frequently, for example, in good *c*-axis films<sup>7</sup>), and the results given here show that they do not cause any degradation of properties. The grain boundary structure of these {103} films, therefore, provides us with a unique opportunity to measure the properties of the 90° [010] twist boundary.

Figure 3 shows resistivity-temperature curves for a {103} YBCO film on (101) LaAlO<sub>3</sub> along the [010] direction as well as that of a typical *c*-axis film made on (100) MgO in the same run. We have measured resistivity,  $T_c$  and  $J_c$  by a four-point measurement. To measure the anisotropy, we patterned cross-shaped bridges with two 40-μm lines. The bridge lines are aligned along [010] and [301] directions of YBCO for {103} film; these are aligned along [100] and [010] directions of the substrate for *c*-axis films.

For the *c*-axis film,  $T_c$  is 86.5 K and the transition width ( $\Delta T_c$ ) is less than 1 K. The resistivity behaviour for the two orthogonal directions is the same as expected for the *a*-*b* twins (no anisotropy). The resistivity at room temperature ( $\rho_{300}$ ) is 195 μΩ cm, the temperature dependence ( $d\rho/dT$ ) is 0.58 μΩ cm K<sup>-1</sup>, and the zero-temperature intercept ( $\rho(0)$ ) is zero. These normal state properties are very close to those of high-quality single crystals for *J* parallel to the *a*-*b* plane.

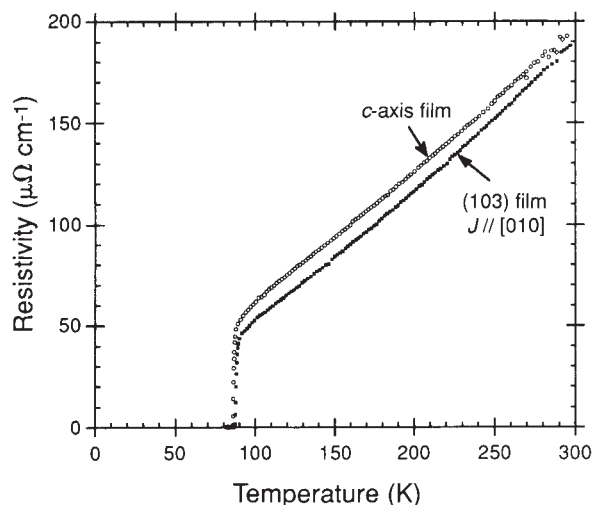


FIG. 3 Resistivity-temperature curves for *c*-axis film on (100) MgO, and for {103} film on (101) LaAlO<sub>3</sub> along the [010] direction.

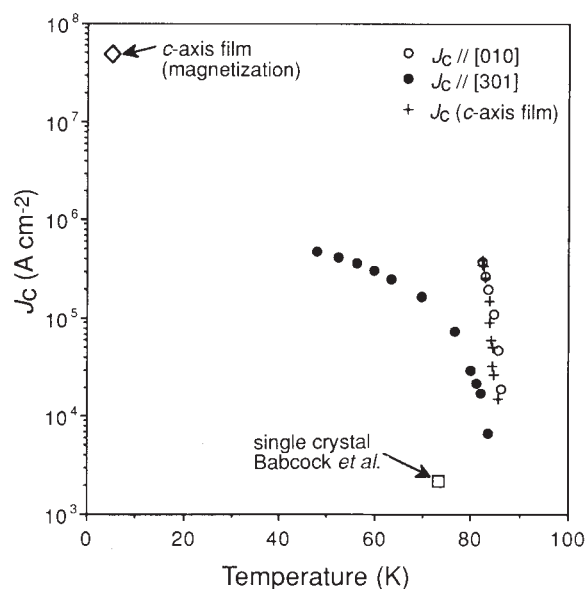


FIG. 4 Critical current densities for *c*-axis film and for {103} film along [010] direction across type A grain boundaries and along [301] direction. The  $J_c$  at 4.2 K was measured by magnetization at near-zero field. The intra-grain  $J_c$  of a flux-grown single crystal<sup>4</sup> is also shown.

For {103} films along the [010] direction,  $T_c$  is 87 K and  $\Delta T_c$  is 1.0 K, almost the same as for the *c*-axis film. Furthermore, the resistivity ( $\rho(T)$ ) is also similar to that of *c*-axis films or single crystals for transport in the *a*-*b* plane. Actually,  $\rho(T)$  from three different runs is consistently slightly lower for {103} films than for the *c*-axis films. The extrapolated intercept gives  $\rho(0) \leq 0$ . Thus there is no evidence for temperature-independent grain boundary resistance, in contrast to the large  $\rho(0)$  in *a*-axis films<sup>8</sup>. Also, the  $\rho(T)$  parallel to [301], which will be reported in detail elsewhere (C.B.E. *et al.*, in preparation), is an order of magnitude higher than that along the [010] direction.

Critical current densities of the film have been measured both by transport on the 40-μm-wide patterned bridge and by the d.c. magnetization method. Our transport  $J_c$  measurements were limited to temperatures near  $T_c$ , because the current source used had a maximum output of 100 mA. The criteria for the transport  $J_c$  was 5 μJ cm<sup>-1</sup>. The magnetization  $J_c$  was measured at 4.2 K at zero field using the Bean<sup>9</sup> approximation.

Figure 4 shows the  $J_c$ - $T$  curves for a *c*-axis film and for a {103} film along two directions. The  $J_c$  for the *c*-axis film, measured by magnetization at 4.2 K, is  $5 \times 10^7$  A cm<sup>-2</sup>. The *c*-axis film we have studied here does not have any high-angle grain boundaries, which was confirmed by an off-axis X-ray scan of the {103} peak. We do not therefore expect any weak-link behaviour in this *c*-axis film. The transport  $J_c$  of {103} films along the [010] direction is, within experimental error, the same as that of the *c*-axis film. The  $J_c$  along the [301] direction is ~30 times lower than along the [010] direction (see Fig. 4).

To summarize, there is no significant difference between *c*-axis films and {103} films measured along the [010] direction with respect to critical current density, and in addition the normal-state conductivity is slightly higher. There are some low-angle grain boundaries electrically in parallel with 90° [010] twist boundaries. The current must flow equally well through both kinds of boundaries in order to account for the similarity of the transport behaviour. A less direct current path length involving the boundaries in other directions, such as [301], is therefore not indicated by either the resistivity or critical current density. We conclude that the 90° twist grain boundaries along the [010] direction cannot be weak links. This unexpected result can be



explained in the following way. The type A twist (Fig. 1) which we are talking about is particularly robust in so far as transport is concerned. Across these boundaries, a single  $\text{CuO}_2$  plane in each grain makes contact with many  $\text{CuO}_2$  planes in the other grain. It is interesting to consider that the  $90^\circ$  [010] twist boundaries may actually improve conductivity by shorting out possible defects in the  $a$ - $b$  plane. This allows transport without having to travel along the highly resistive  $c$ -axis. The  $90^\circ$  [010] twist boundary also may be advantageous in situations where electrical contact is made only to a surface layer. Although there is some antisite cation disorder between barium and yttrium, it seems reasonable that the energy band structure remains essentially unaltered across the  $90^\circ$  [010] twist boundary, as the copper and oxygen atoms all remain in their correct positions.  $\square$

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## A gas-lens telescope

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A GAS lens is capable of focusing light if the temperature, and therefore refractive index, of a gas is made to vary across an optical aperture in a suitable manner. Practical applications of gas lenses were pursued thirty years ago<sup>1,2</sup> in the context of power transmission by laser beams, but little work has been done since then. It was recently shown, however, that a gas lens can focus a laser beam well enough to drill holes in a metal sheet<sup>3</sup>, and it has been argued<sup>4</sup> that gas lenses are 'varifocal' devices with negligible dispersion from ultraviolet to infrared wavelengths, and that they may be able to transmit and focus more powerful laser beams than conventional lenses can cope with. They may therefore find some application in laser-driven thermonuclear experiments<sup>5</sup>. Here we describe another application of gas lenses: telescopes. We have constructed a simple gas lens telescope, and have used it to make images of the Sun and the Moon.

One of the simplest gas lenses consists of an electrically heated pipe through which air is drawn (see Fig. 1a). Air at room temperature in the centre of the pipe has a higher refractive index than the warm air at the boundary. A refractive index gradient results which brings rays of laser light or rays from a distant object to a sharp focus. Typical operating parameters are: pipe wall temperature  $T_w = 45^\circ\text{C} = 318\text{ K}$ ; pipe length  $l = 0.2\text{ m}$ ; tube radius  $r = 4\text{ mm}$ ; focal length  $f = 1\text{ m}$ . (The gradient bends the light in the same way as that above a hot road surface producing a mirage. Essentially a gas lens is a cylindrical mirage device).

An order-of-magnitude calculation of the expected focal length shows how  $f$  depends on  $T_w$ ,  $l$  and  $r$ . This rough calculation is valid for vertical, horizontal, static and spinning gas lenses. Assume that the air at the centre of the pipe is at room

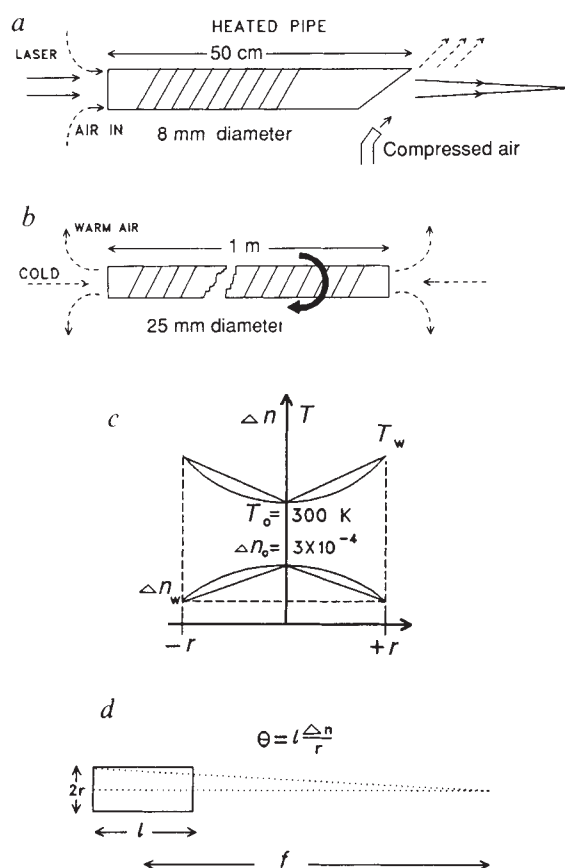


FIG. 1 A simple gas lens is made by passing air through a heated tube (a), so that the refractive index varies across the aperture. Spinning the tube (b) stabilizes the air-flow and allows a larger aperture. Focusing can be modelled (c) by assuming a simple linear gradient of temperature and refractive index across the tube, or in a more sophisticated treatment by using a parabolic gradient. The relationship between the telescope's dimensions and its optical properties is shown in d.

temperature  $T_0 = 300\text{ K}$  and the air at the wall is at  $T_w$ . As a first approximation, we take the temperature gradient to be linear (see Fig. 1c). The refractive index of air at standard temperature and pressure differs from unity by the small amount  $\Delta n_0 = 3 \times 10^{-4}$ . The difference  $\Delta n$  is proportional to the air density and therefore inversely proportional to absolute temperature if the air in the tube is at atmospheric pressure. Thus

$$\frac{\Delta n_0}{\Delta n_w} = \frac{T_w}{T_0}$$

where  $1 + \Delta n_w$  is the refractive index at the wall. So

$$\frac{\Delta n_0 - \Delta n_w}{\Delta n_w} = \frac{\Delta n}{\Delta n_w} = \frac{T_w - T_0}{T_0} = \frac{\Delta T}{T_0}$$

where  $\Delta n$  and  $\Delta T$  are the total differences in refractive index and temperature across the tube, respectively. Now the angle of deflection  $\theta$ , of a paraxial ray is proportional to the length,  $l$ , of the tube and the refractive index gradient:

$$\theta = l \frac{\Delta n}{\Delta r}$$

Therefore in our case  $\theta = l \Delta n / r$ , and the outer rays intersect the axis at a distance  $f$  such that  $r = f\theta$ . Thus

$$f \approx 10^6 r^2 / (l \Delta T)$$

A slightly more sophisticated treatment taking the parabolic refractive index profile of Fig. 1c (which produces a real focus)

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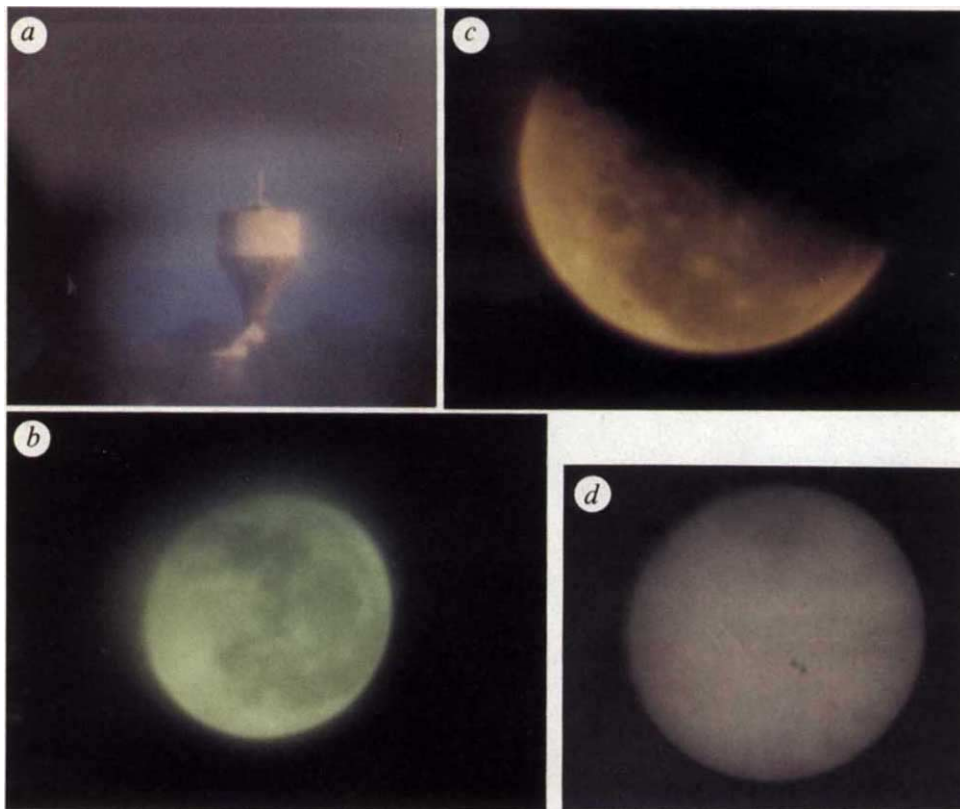


FIG. 2 Gas telescope images of *a*, a water tower at a distance of 5 km; *b*, the Moon; *c*, lunar craters, visible near the terminator; *d*, Sun spots taken during solar maximum.

yields<sup>3</sup>

$$f = \frac{\cot(\sqrt{Al})}{\sqrt{A}} \quad \text{with} \quad A = \frac{6 \times 10^{-4} \Delta T}{r^2 T_w}$$

which for the above example gives  $f \approx 0.9$  m. In fact this formula is only correct if  $l$  is replaced by  $l'$ , an effective pipe length that takes aerodynamic effects into account<sup>6</sup>.

Provided that the pipe diameter is less than a centimetre, such blown or sucked air lenses work well and can be used to image distant objects<sup>7</sup>. Figure 2*a* shows a water tower at 5 km imaged through an 8 mm-diameter sucked air lens. Unfortunately such lenses have a very small field of view and low speed. With static horizontal lenses in which the diameter exceeds 1 cm, vertical convection currents destroy the laminar flow and with it the focus<sup>2</sup>. One solution of this problem is to inject compressed air or gas and to spin the pipe<sup>8</sup>. We have tested many types of spinning gas lenses and have recently found that a 2.5-cm-diameter heated pipe spun at 30 Hz, without compressed air injection, works very well and gives as good a focus as any large-aperture gas lens. Our lens operates by centrifuging warm air out of the ends and drawing cold air down the axis (see Fig. 1*b*).

We have constructed a simple 'gas telescope' consisting of a computer-steered plane mirror, a 1-m-long spinning-pipe gas lens and a lensless camera. Figure 2*b* and *c* shows pictures of the moon and lunar craters (mirror image), and Fig. 2*d* shows sun spots taken during solar maximum.

It has been suggested that 'gas telescopes' will never make its mark in astronomy, but we contend that gas lenses are in their infancy. We have already extended the useful aperture from 1 cm to 5 cm. Gas lenses have focused CO<sub>2</sub> laser beams to drill holes in 1-mm-thick steel sheet; they have been used to generate laser plasmas<sup>9</sup> and have been operated together with phase-conjugate mirrors<sup>10</sup>. A different type of gas lens (a helium-filled bag) has recently been proposed as a remedy to the aberrations of the Hubble Space Telescope<sup>11</sup>. Improvements in gas lens design should widen the range of possible applications. As

mentioned above, gas lenses are able to transmit more laser power than glass lenses. The damage threshold intensity for short pulses through glass or germanium lenses is  $\sim 1 \text{ GW cm}^{-2}$ . Gases break down at intensities two orders of magnitude higher. It goes almost without saying that gas lenses, unlike solid lenses, are indestructible.

A promising field of research is satellite propulsion by ground-based laser<sup>12</sup>. Gas lenses with their long and variable focal length and high power threshold could be developed to direct the beam. No ordinary lens or mirror system will handle the required twenty 200 kJ pulses per second for ten minutes or more.

Finally, we wish to point out that in the absence of convection currents gas lenses are expected to work well in a zero-gravity environment. A study of gas lenses and telescopes in space has been proposed to NASA (C. R. Phipps, personal communication). □

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# Molecular design based on recognition at inorganic surfaces

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**MOLECULAR** recognition at inorganic surfaces has the potential to provide control over crystal growth processes. For organic surfaces, molecules that influence crystallization<sup>1-3</sup> can be derived by rational modification of host molecules to give the stereochemistry required by the surface structure. This approach is of little use for inorganic systems, however, because the relatively simple stereochemistries of the surfaces and ions concerned allow little scope for similar manipulation. Previous work<sup>4-6</sup>, therefore, has been essentially phenomenological. Here we show that a detailed understanding of recognition processes at inorganic surfaces can nevertheless lead to the rational design of surface-active molecules. We have used crystal morphological characteristics to deduce the nature of the surface binding sites of diphosphonates on barium sulphate crystals, and have thereby been able to design new surface-active molecules with improved efficacy.

This work was stimulated by the need for more effective chemicals to control inorganic scale ( $\text{CaSO}_4 \cdot n\text{H}_2\text{O}$ ,  $\text{CaCO}_3$ ,  $\text{BaSO}_4$ ) generated in water-treatment systems, particularly in off-shore oil production where scale formation in pipelines and porous oil-bearing strata threatens operations. Effective antiscalants are molecules that inhibit the crystal growth of inorganic phases by blocking growth sites, particularly on the fastest growing surfaces.

The experimental morphology of barytes (barium sulphate) was determined from synthetic crystals, prepared under conditions mimicking off-shore environments, to be (001) rhombic plates bounded by (210) side faces. This simple morphology is identical to that formed in single-cell organisms<sup>7</sup> and indicates that growth is fastest in the [001] zone. From a range of carboxylates, phosphonates and sulphonates tested, only one group of molecules showed any specific effect on this morphology<sup>8</sup>. These were diphosphonates in which the phosphorus atoms were linked by a three-atom chain, for example, amino dimethylene diphosphonic acid. These molecules had a pronounced effect on crystal growth. With increasing concentration, the morphology (Fig. 1) progressed from rhombs with pinched corners ((011) face) through hexagons ((010) face) to disks (entire [001] zone). These experiments were conducted at pH 7; below pH 5 no effects were observed.

These data allowed us to elucidate the key recognition elements operating at the growing barytes surfaces. First, the inactivity of sulphonates and carboxylates, together with the influence of decreasing pH, showed that only doubly charged anions (phosphonates above pH 5) are recognized by the growing surfaces. Second, the morphologies indicated that the binding site for the phosphonate must be located on the (011) surface, which is built up from alternate layers of barium and sulphate ions. The separation (S-S) of neighbouring like-oriented sulphate ions in this surface is 5.6 Å (Fig. 2). The energy-minimized conformation of the fully deprotonated amino dimethylene diphosphonate ion, calculated by MOPAC,<sup>9</sup> gave an inter-phosphorus distance of 5.7 Å, which is a good match for the S-S separation. Taken together, these data are consistent with simultaneous replacement of two tetrahedral sulphate ions by the diphosphonate (Fig. 2). It is these electrostatic, geometric and stereochemical features that give this diphosphonate motif its efficacy.

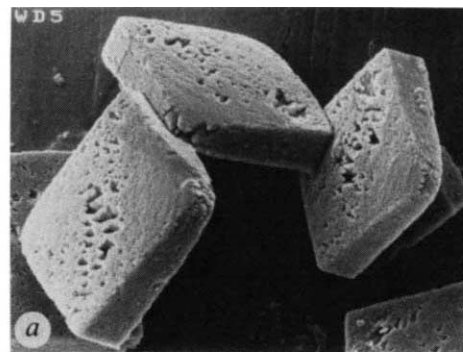
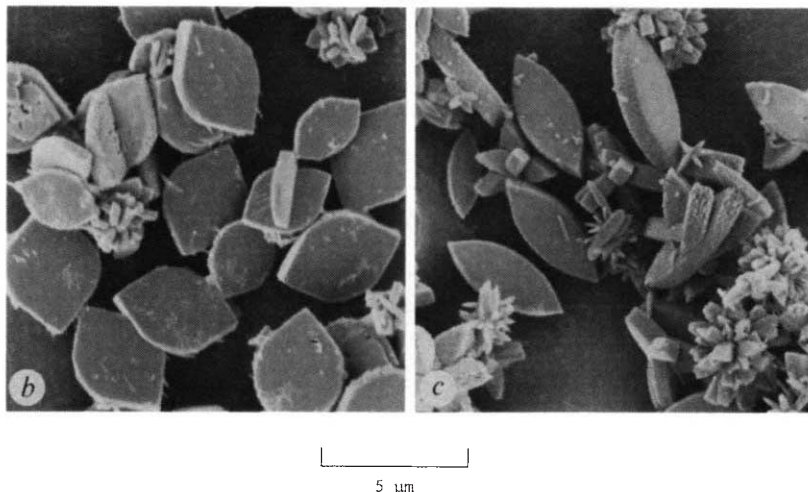


FIG. 1 Morphology of  $\text{BaSO}_4$  in the presence of amino dimethylene diphosphonic acid. *a*, 0.024 mM, *b*, 0.048 mM, *c*, 0.096 mM.



With this understanding we were able to build on previous work<sup>4,6</sup>, in which the recognition properties of only pre-existing molecules were explored, and, in contrast, formulate a strategy for molecular design. This was based on the concept that molecules containing more than one active motif, capable of simultaneously binding to several surface sites, would have enhanced binding energies and offer a superior steric barrier, making them more effective inhibitors than molecules containing a single motif. Two active groups occupying adjacent binding sites have a N-N separation of 9 Å (Fig. 2) and this defines the requirement for linking motifs. From molecular modelling (Chem-X molecular modelling suite; developed and distributed by Chemical Design Ltd, Oxford), and considering the stereochemistry at the nitrogen centres, it was clear that in the case of a dimer the shortest link was a seven-carbon-atom chain ( $C_1-C_7 = 7.4$  Å).

To substantiate these ideas, we used the Mannich reaction<sup>10</sup> to convert the diamines  $NH_2(CH_2)_nNH_2$  ( $n = 6-9$ ), 2,2'-(ethylene dioxy) diethylamine and 3,3'-(ethylene dioxy) dipropylamine (from Aldrich, Fluka and Fluorochem Ltd, respectively) to their corresponding amino phosphonic acids. We evaluated each of these acids for its effect on the morphology of barytes crystals by scanning electron microscopy, and for its ability to prevent precipitation by measuring the  $Ba^{2+}$  concentration in solution (ICP) after 4 h. (The effects of the additives were compared at equivalent concentrations of the active motif.) Figure 3 shows a summary of the latter data. The molecule 1,6 hexamethylene di(amino bismethylene diphosphonate), which according to our model cannot span two binding sites, showed no improvement over the molecule with the single motif. Its

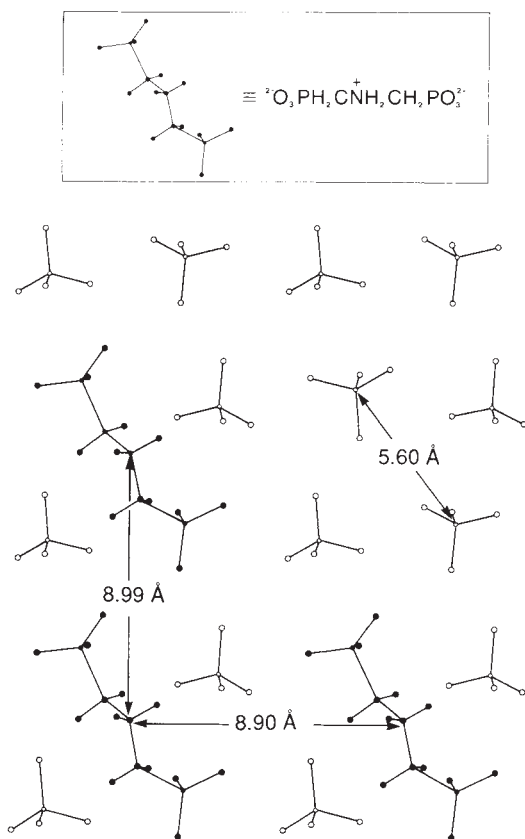


FIG. 2 Projection of the  $BaSO_4$  structure normal to the (011) sulphate layer, showing the relationship between neighbouring like-oriented sulphate ions and between diphosphonate ions replacing sulphate ions in adjacent binding sites.

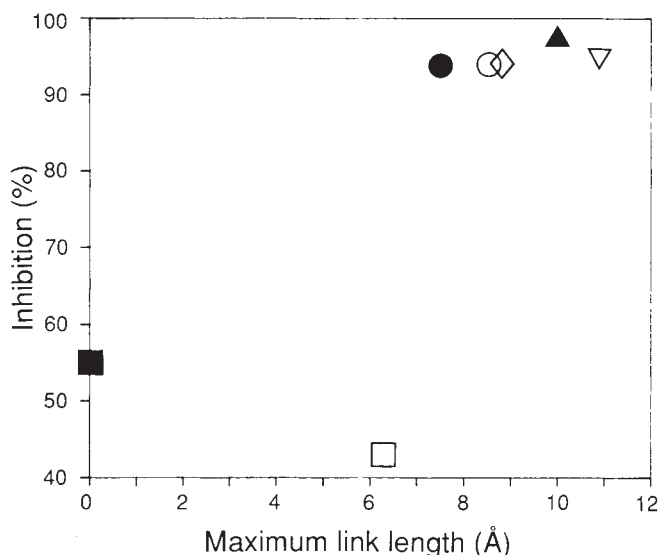


FIG. 3 Relationship between the inhibitory power of variously linked amino dimethylene diphosphonic acid dimers and the maximum length of the link. The result for the unsubstituted motif is recorded for comparison. Concentration of the active motif is 0.96 mM in all cases. ■,  $HN(CH_2PO_3H_2)_2$ ; □,  $(H_2O_3PH_2C)_2N(CH_2)_6N(CH_2PO_3H_2)_2$ ; ●,  $(H_2O_3PH_2C)_2N(CH_2)_7N(CH_2PO_3H_2)_2$ ; ○,  $(H_2O_3PH_2C)_2N(CH_2)_8N(CH_2PO_3H_2)_2$ ; ◇,  $(H_2O_3PH_2C)_2N(CH_2)_8N(CH_2PO_3H_2)_2$ ; ▲,  $(H_2O_3PH_2C)_2N(CH_2)_9N(CH_2PO_3H_2)_2$ ; ▽,  $(H_2O_3PH_2C)_2N(CH_2)_3O(CH_2)_2O(CH_2)_3N(CH_2PO_3H_2)_2$ .

reduced performance is presumably the balance of two opposing effects: the first should effectively reduce the performance by half, as two motifs are removed for each molecule bound; the second should improve the performance because the unbound part of the molecule acts as a steric barrier.

The performance of those molecules with linkages greater than 7.4 Å, however, was considerably enhanced (as predicted) with effectively all precipitation being inhibited. The morphologies for all these additives showed disrupted growth in the [001] zone at concentrations an order of magnitude less than for the monomers. Both these results were in agreement with the proposed binding model in which two diphosphonate motifs simultaneously access surface binding sites.

The obvious continuation of this work is to consider dimers with rigid links and then polymers. The advantage of the former would be to minimize the energy barriers arising from the conformational requirements of the surface/molecule interaction. We are exploring the possibility of using linking groups based on benzophenone, dibenzofuran and carbazole. For polymers, synthesized by graft reactions or direct polymerisation, the goal will be to create tailored macromolecules with even higher degrees of surface specificity. □

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# Do bidecadal oscillations exist in the global temperature record?

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**MODELS predict<sup>1-3</sup> that increasing atmospheric concentrations of greenhouse gases will result in an increase in the global mean temperature over the next few decades and beyond. It is therefore important to be able to distinguish a warming trend from natural variability in the time series of global temperatures. Recently, Ghil and Vautard<sup>4</sup> applied singular spectrum analysis to a record of global surface air temperatures, and identified a secular warming trend and a small number of oscillatory modes. The oscillations had interdecadal periods of 21 and 16 years (attributed to changes in the extratropical ocean circulation), and interannual periods of 6 and 5 years (attributed to the El Niño/Southern Oscillation). Here we re-analyse the data by considering various lengths of the temperature record, and we apply singular spectrum analysis to five other temperature records (two global, three hemispheric). Our results offer no support for the presence of bidecadal oscillations in the global surface temperature records.**

Because of the importance of the problem, considerable efforts have been made to extract information about trends, periodicities, natural variability and noise from the records<sup>5-8</sup>. Ghil and Vautard (GV hereafter)<sup>4</sup> applied singular spectrum analysis (SSA) to one of the available records. This approach<sup>9,10</sup>, which is fully non-parametric, considers  $M$  lagged copies of a centred time series  $X(t)$  sampled at equal intervals  $\tau$ ,  $X_i = X(t_0 + i\tau)$ ,  $i = 1, N$ , and estimates the eigenvalues  $\lambda_k$  and eigenvectors  $\rho_k$  of their covariance matrix  $C$  (here  $1 \leq k \leq M$ ). They (GV) call the eigenvectors empirical orthogonal functions (EOFs) and the coefficients  $a_k$  involved in the expansion of each lagged copy, principal components (PCs). EOFs are of interest when among  $k$  eigenvalues there exist a number of distinct ones whose magnitude is appreciable, whereas the rest are close to zero. In such cases this would be a strong indication of 'deterministic' parts in the subspace of eigenmodes with the rest of the modes acting as noise. Thus, this method can be used to separate signal from noise. In an earlier study Vautard and Ghil<sup>11</sup> observed that pairs of high-variance eigenvalues  $\lambda_k = \lambda_{k+1}$  are associated with oscillatory phenomena (both the corresponding EOFs and PCs are in quadrature with each other).

In their analysis, GV extracted the following oscillatory modes: an oscillation having a period of nearly 20 years (EOFs 3 and 4), an oscillation of 6 years (EOFs 11 and 12) and an oscillation of 5 years (EOFs 6 and 7). The five- and six-year periodicities are attributed to the well-documented El Niño/Southern Oscillation (ENSO). Speculation concerning the 20-year oscillation centres on possible changes in ocean circulation. From these results, GV proceed to reconstruct the global climate record and to investigate the issue of detecting a greenhouse warming signal in the record. They conclude that, as a consequence of the bidecadal oscillation, the warming signal will not be detectable for at least one or two more decades.

Our interest in this important method of analysing the data is the effect of the record length on the results. We thus considered the UK global surface air temperature record of Jones *et al.*<sup>12</sup> and repeated SSA for various record lengths. This record was provided by the Oak Ridge National Laboratory, and it covers the period 1861–1990. Shown in Fig. 1 are the eigenvalues of the lag-covariance matrix  $C$  against order for the entire record ( $N = 130$ ), for the record from 1881–1990 ( $N = 110$ ) and for the record from 1901–1990 ( $N = 90$ ). For all the following analyses, we used  $M = 30$ . As did GV, we found no significant changes

to any of the results when  $M$  was varied between 25 and 50. For any length we observe that the first two eigenvalues ( $\lambda_1$  and  $\lambda_2$ ) considerably exceed the rest, explaining 35–65% of the variance. The second two eigenvalues ( $\lambda_3$  and  $\lambda_4$ ) are quite close to the noise floor, and account for only 7–11% of the variance. It is interesting to note that our Fig. 1 does not exactly reproduce GV's corresponding figure based on the same but somewhat larger record (1854–1988,  $N = 135$ ). The reason for the slight mismatch will become clear below.

Shown in Fig. 2 are the corresponding leading eigenvectors (EOFs 1–4) for the different length records. We observe that the estimation of EOFs 1 and 2 is robust; it varies only slightly with record length. EOFs 1 and 2 correspond to the trend in the temperature record. In contrast, however, the estimation of EOFs 3 and 4 is not robust. If the entire record ( $N = 130$ ) is considered, EOFs 3 and 4 are an oscillatory pair in quadrature with each other and have a period of  $\sim 20$  years, but for  $N = 110$  and  $N = 90$  this is not the case. Instead, we begin to see an oscillatory pair having a period between five and six years. We note that for  $N = 110$  and  $N = 90$ , no higher EOFs (not shown) indicate the 20-year oscillation. The result is not surprising, as the eigenvalues corresponding to EOFs 3 and 4 are clearly very near the noise floor.

From these results, we raise the question of whether a minimum  $N$  is required for delineation of the bidecadal oscillations. To answer this question, we repeat the analysis using records that are shortened from the end (most recent years), rather than from the beginning (earlier years) as was done above. Results are shown in Fig. 3. We now find that, along with the estimation of EOFs 1 and 2 (not shown), the estimation of EOFs 3 and 4 is robust with bidecadal oscillations present for all data lengths considered. Thus the comparison of Figs 2 and 3 reveals that it is not the length of the record, but the inclusion of data points before roughly 1881 that makes the difference. Include the earliest years in the analysis and you will detect a bidecadal oscillation, but exclude them and it disappears.

Other global surface air temperature records tell a similar story. Estimates of EOFs 3 and 4 from SSA on the USA global data set<sup>13</sup>, on the IPCC global time series<sup>14</sup> and on the USSR data set<sup>15</sup> are shown in Fig. 4. The USA record extends from 1880 to 1987 ( $N = 108$ ). The IPCC record extends from 1961 to 1990 ( $N = 130$ ; the 1990 value of the IPCC data was supplied by D. E. Parker). The USSR data set represents annual mean surface air temperatures in the Northern Hemisphere, and it covers the period 1891–1987 ( $N = 97$ ). Estimates of EOFs 1 and 2 from all data sets (not shown) are close to those estimated from the UK data set. In both the USA and the USSR sets,

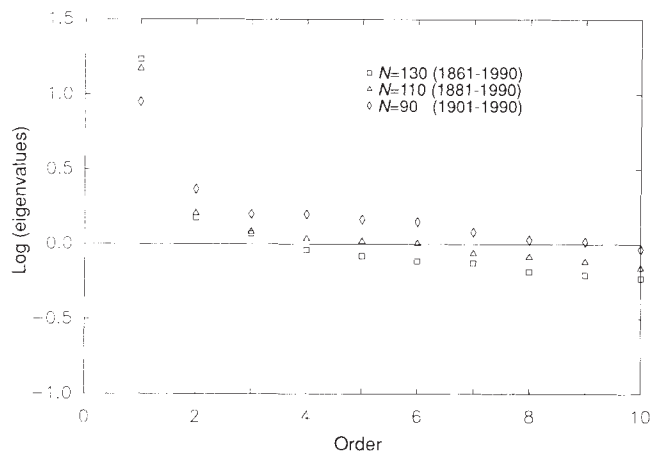


FIG. 1 The estimated eigenvalues,  $\lambda_k$ , of the lag-covariance matrix  $C$  ( $M = 30$ ) calculated for various lengths of the global surface temperature record<sup>12</sup>. Only the first two eigenvalues extend above the noise floor.

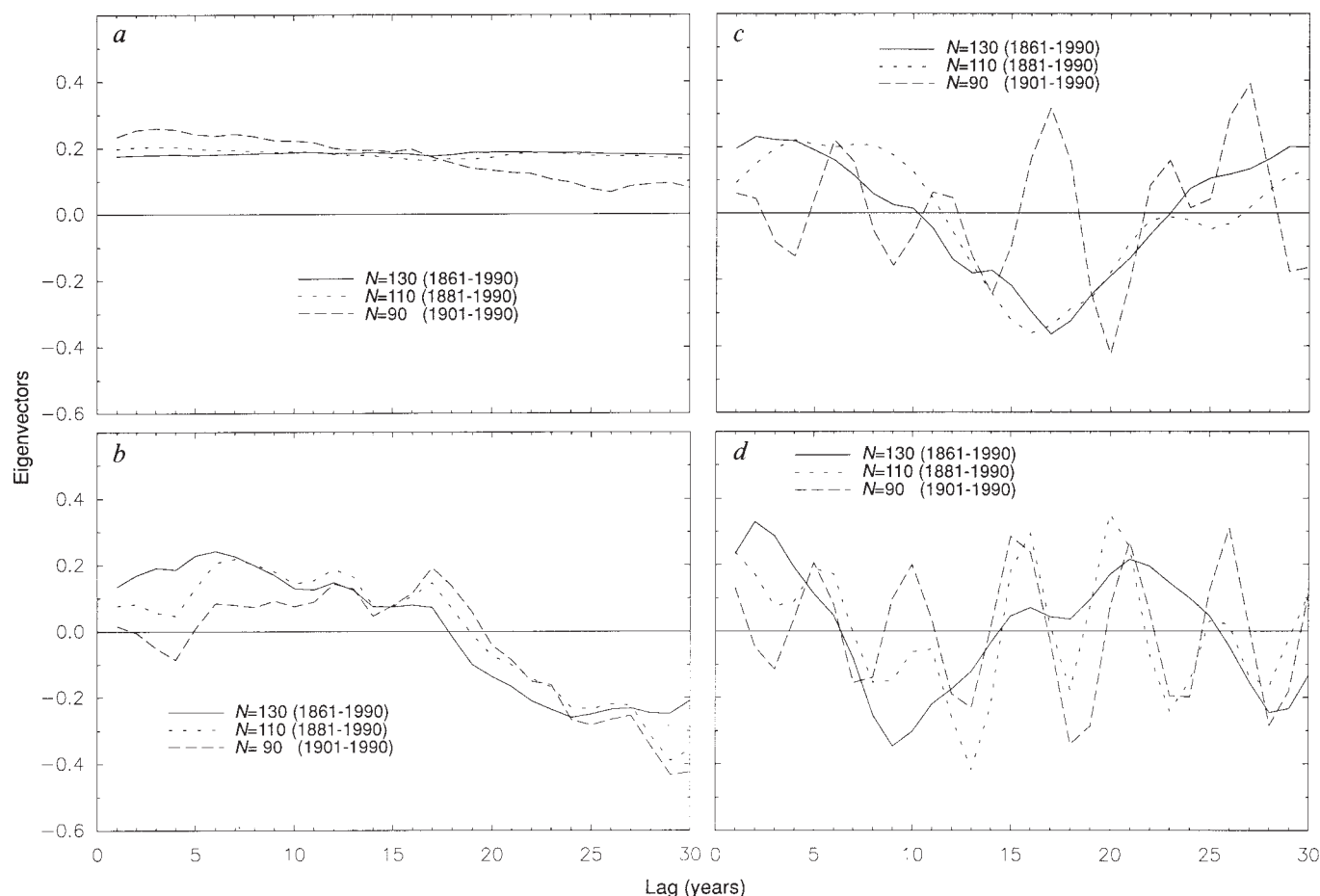


FIG. 2 *a*, The first eigenvector (EOF 1) of the lag-covariance matrix  $\mathbf{C}$  ( $M=30$ ) computed for various lengths of the record<sup>12</sup>. All records end with the year 1990. *b*, As in (*a*), but for EOF 2; *c*, as in (*a*) but for EOF 3, *d*, as in (*a*) but for EOF 4. Here EOFs 3 and 4 are a pair in quadrature and having a period of  $\sim 20$  years only for  $N=130$  (1861–1990). EOFs 1 and 2 correspond to the trend and are virtually unchanged for different record lengths.

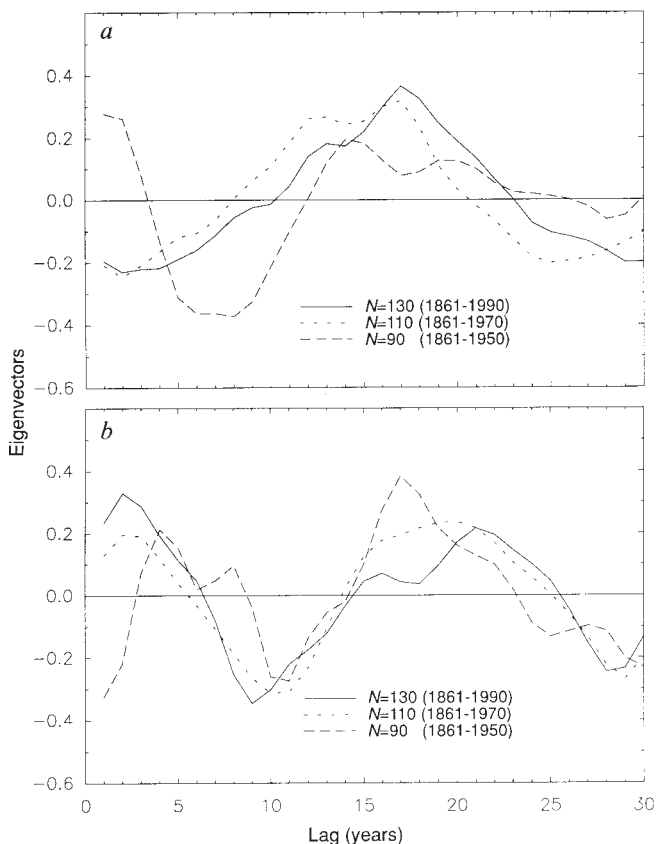


FIG. 3 *a*, EOF 3 estimated from the lag-covariance matrix  $\mathbf{C}$  ( $M=30$ ) using various lengths of the record<sup>12</sup>. Now all records begin with the year 1861. *b*, As in (*a*) but for EOF 4. Here EOFs 3 and 4 are an oscillatory pair in quadrature and with a period of  $\sim 20$  years for all record lengths.

which do not include any values before the 1880s, the 20-year oscillatory pair is not apparent (if anything, EOFs 3 and 4 indicate a five- to six-year oscillatory pair). The results from the IPCC record are similar to the results obtained from the UK set: the 20-year oscillation is not robust. It exists when the whole record is considered, but it is absent when somewhat smaller periods excluding early values, such as the period 1891–1990 ( $N=100$ ) shown in Fig. 4, are considered. As the USSR set is for the Northern Hemisphere, we also repeated the analysis for the northern hemispheric values of the USA and UK records that were available to us. Both data sets are in agreement: each reveals a five- to six-year periodicity associated with ENSO and no bidecadal oscillation when data before 1880 are not included. In addition, we note that no bidecadal oscillation is found in any of the higher EOFs.

In the past, other investigators have come across a peak at 21–23 years but none of them refers to a global surface air temperature record. For example, there is a pronounced peak at 23 years in the maximum entropy spectrum of a 300-year



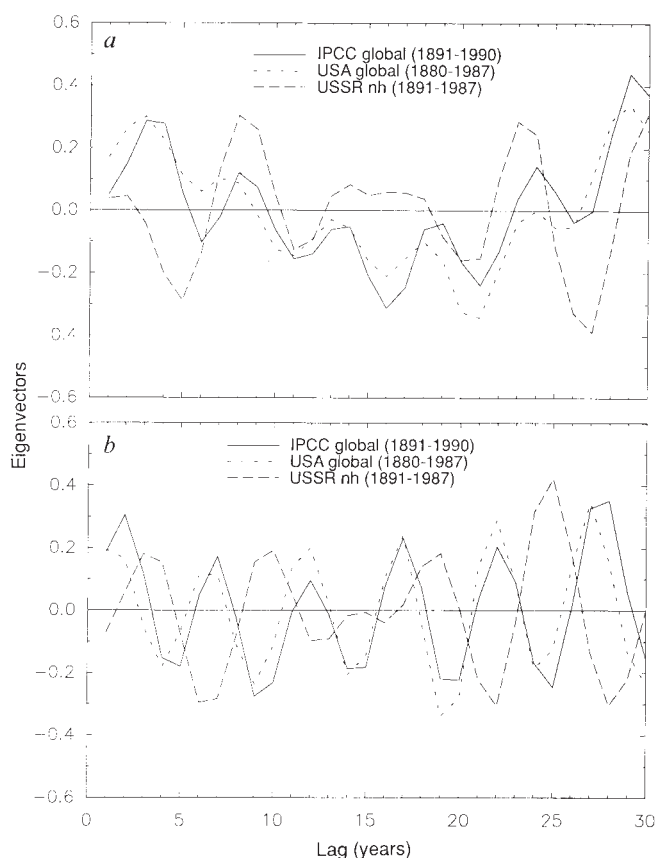


FIG. 4 *a*, EOF 3 and *b*, EOF 4 estimated from the lag-covariance matrix **C** ( $M=30$ ) for the USA global temperature record<sup>13</sup> (1880–1987), the IPCC record<sup>14</sup> (1891–1990) and for the USSR data set<sup>15</sup> (1891–1987; nh, Northern Hemisphere). Note the absence of bidecadal oscillations.

temperature record<sup>16</sup> and a 21.8-year peak is contained in the Fourier transform of global marine air temperature data<sup>17</sup> covering the period 1856–1986. The 23-year peak refers to just a small area in central England, and the 21.8-year peak refers to marine data only. Although both important, their statistical significance with respect to their connection to global surface air temperature records is not straightforward and was never established.

We therefore conclude that it is only the values from before the 1880s that cause the SSA to delineate bidecadal oscillations in global surface air records. A record of length  $N=100$  years starting at 1861 would show bidecadal oscillations, but a record of equal length ending today would not. The great dependence of the results of GV on the data before 1880, together with the poor quality and density of the data during this period (a fact that GV recognized) and possibly the fact that SSA is designed for stationary data (which the temperature records most surely are not) make the existence of bidecadal oscillations in the global surface temperature records, at best, questionable.

The idea that low-frequency variations in the climate system can be traced to the thermal inertia of the ocean is not questioned. Certainly ENSO is an example. But without a physical model of the mechanisms involved, supported by other evidence from the data, the bidecadal oscillation cannot be considered significant at this time. □

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## Picritic glasses from Hawaii

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ESTIMATES of the MgO content of primary Hawaiian tholeiitic melts range from 8 wt% to as high as 25 wt% (refs 1, 2). In general, these estimates are derived from analysis of the whole-rock composition of lavas, coupled with the compositions of the most magnesian olivine phenocrysts observed. But the best estimate of magma composition comes from volcanic glass, as it represents the liquid composition at the time of quenching; minimal changes occur during the quenching process. Here we report the discovery of tholeiitic basalt glasses, recovered offshore of Kilauea volcano, that contain up to 15.0 wt% MgO. To our knowledge, these are the most magnesian glasses, and have the highest eruption temperatures (~1,316 °C), yet found. The existence of these picritic (high-MgO) liquids provides constraints on the temperature structure of the upper mantle, magma transport and the material and thermal budgets of the Hawaiian volcanoes. Furthermore, picritic melts are affected little by magma-reservoir processes, and it is therefore relatively straightforward to extrapolate back to the composition of the primary melt and its volatile contents.

Tholeiitic basalt glass sand was recovered in a box core near the base of the submarine East Rift Zone (Puna Ridge) of Kilauea volcano at 19° 59.4' N, 154° 26.4' W at a depth of 5,500 m during a US Geological Survey cruise in 1988 (ref. 3). The sandy layers in the core contain a variety of unaltered submarine erupted glasses ( $S>250$  p.p.m.), crystal fragments (mainly olivine), crystalline rock fragments, devitrified glass fragments and rare radiolarians. Most glass sand grains lack any palagonite alteration rim, suggesting they are younger than a few thousand years. Glass sand grains (~200 analysed by microprobe; most <300 µm in size) from the surface layer and sandy layers at 9, 15, 21, 26, 29, 31 and 41 cm are compositionally similar to glass on pillow lavas from the Puna Ridge which generally contain MgO < 7 wt% (refs 4–7). Rare grains from the surface layer and from layers at 26, 31 and 41 cm, however, have MgO > 8 wt% as do common grains from the 55–58 cm layer. The compositions of most of these grains, like the pillow rind glasses from the Puna Ridge<sup>7</sup>, fall between prehistoric Kilauea and Mauna Loa whole-rock compositions<sup>8</sup>. The CO<sub>2</sub> contents of seven of these high-MgO, high-S glasses are 69–107 p.p.m. (Table 1), which, assuming that these magmas were saturated with respect to CO<sub>2</sub> at the pressure of eruption (as documented for pillow lavas erupted along the Puna Ridge<sup>6</sup>), correspond to eruption depths of ~1,800–2,800 m. A single analysed high-MgO glass, with low S and H<sub>2</sub>O contents and CO<sub>2</sub> below the detection limit (~20 p.p.m.), experienced low-pressure degassing and was probably erupted at <500 m depth. Such unaltered tholeiitic glasses must be from Kilauea, as Mauna Kea, whose submarine rift is nearby, last erupted tholeiitic basalt several hundred thousand years ago, and Mauna Loa has no submarine rift on the north side of the island.

The unaltered glasses in the sandy layer at 55–58 cm are dominated by a vesicular glass containing 7.3–7.7 wt% MgO, but lower SiO<sub>2</sub> and higher TiO<sub>2</sub>, K<sub>2</sub>O, P<sub>2</sub>O<sub>5</sub> and FeO (Table 1) than most Kilauea glasses with similar MgO contents. This glass is similar to the 1959 Kilauea Iki lava<sup>8</sup>, which is an unusual Kilauea composition apparently formed by a slightly smaller degree of partial melting than most Kilauea basalts. Associated glass grains range from an extreme differentiate containing ~1 wt% MgO and 59 wt% SiO<sub>2</sub>, to moderately differentiated

tholeiitic glasses similar to pillow basalts from Puna Ridge<sup>4–7</sup>, to the high-MgO glasses that are the subject of this paper.

Glasses from the core and the pillow lavas with MgO < 7 wt% define a multiphase fractionation trend characterized by decreasing CaO (Fig. 1a) and Al<sub>2</sub>O<sub>3</sub> with decreasing MgO produced by crystallization of olivine, clinopyroxene and plagioclase in proportions of roughly 1:5:5 (ref. 6). The compositions of the pillow-rind glasses and their enclosed phenocrysts are discussed in detail elsewhere<sup>6,7</sup>. Glasses from the core with MgO > 7 wt% (preferentially selected for analysis) define olivine fractionation curves (continuous fractionation of equilibrium olivine), which for FeO (Fig. 1b) and SiO<sub>2</sub> are curved, in contrast to linear olivine control (accumulation) lines. The high-MgO glasses with high S contents plot roughly along olivine fractionation curves, whereas the more degassed (lower S) glasses have nearly constant FeO and plot along an olivine control line. It is unclear why the low-S melts plot along an olivine control line. The scatter in the data is beyond that expected from analytical imprecision and suggests that the glasses are not cogenetic; presumably these variations are caused by differences in partial melting conditions or source compositions. The high-MgO glasses constitute ~1% of the sand grains in the 55–58 cm layer, and less in the other layers, but are important because they contain up to 15.0 wt% MgO (Table 1) and demonstrate that some primary magmas at Kilauea are at least this rich in MgO. Based on an experimentally calibrated MgO-geothermometer for Kilauea glass compositions<sup>9</sup>, these glasses have calculated eruption temperatures as high as 1,316 °C.

Many high-MgO glasses contain olivine microphenocrysts as forsterite-rich as Fo<sub>90.7</sub> (Table 1). In contrast, olivine phenocrysts are generally more fayalitic than Fo<sub>88.6</sub> in subaerially erupted lavas<sup>10</sup> and Fo<sub>90.3</sub> in submarine erupted pillow lavas<sup>7</sup>. Few of the olivine cores are in equilibrium with the surrounding glass (Fig. 2). One interpretation of the olivine-glass data is that the melts having Mg#s (defined in Table 1) of 65–70 are mixtures of melts with Mg#s up to ~72 and their enclosed equilibrium olivine crystals (<Fo<sub>90.7</sub>) and melts with Mg#s as low as ~64 and their enclosed equilibrium olivine crystals (>Fo<sub>84.5</sub>).

Primary Hawaiian tholeiitic melts have been proposed to contain from 8 wt% MgO (ref. 1) to 20–25 wt% MgO (ref. 2) with most estimates<sup>11–13</sup> between 12 and 17 wt% MgO. Before this study, the most MgO-rich glasses (melt compositions) known from Hawaii were tephra and inclusions in olivine from Kilauea containing 10.0 wt% MgO (refs 10, 14), and submarine glasses from Haleakala containing 10.2 wt% MgO<sup>15</sup>. The most forsteritic olivine and the trend lines of the high-S glasses can be used to estimate the composition of magma in equilibrium with that olivine, which we propose represents a primary tholeiitic melt composition. Necessary assumptions include the ratio of Fe<sup>2+</sup>/Fe<sub>total</sub> in the melt and the distribution coefficient  $K_D$  for Fe–Mg between olivine and melt. We have used a ratio of Fe<sup>2+</sup>/Fe<sub>total</sub> = 0.9,  $K_D$  = 0.3, and the least-squares fit through the dry normalized glass data on an MgO–FeO diagram. The remainder of the estimated average primary melt composition is calculated from the least-square fits to the other oxides on MgO-variation diagrams and the estimated MgO content of the average equilibrium melt. The resultant normalized primary melt composition for Kilauea volcano contains 17.1 wt% MgO (Table 1) and has an estimated temperature of 1,358 °C. The common occurrence of olivine xenocrysts as forsterite-rich as Fo<sub>90</sub> in olivine-rich pillow lavas from the Puna Ridge<sup>7</sup> supports the idea that high-MgO melts are the dominant magma type introduced into Kilauea volcano from the mantle.

The NiO contents of Fo<sub>90.7</sub> microphenocrysts are 0.55 wt% as compared with 0.38 wt% in Fo<sub>90–91</sub> olivine in lherzolite xenoliths from Hawaii and worldwide (including some analysed on the same instrument with the same data reduction procedure). Using a distribution coefficient for Ni between olivine and melt ( $D_{Ni}$ ) of 6.4 calculated<sup>16</sup> for a melt with 17.1 wt% MgO, the average primary magma contains 680 p.p.m. Ni (0.086 wt% NiO), in

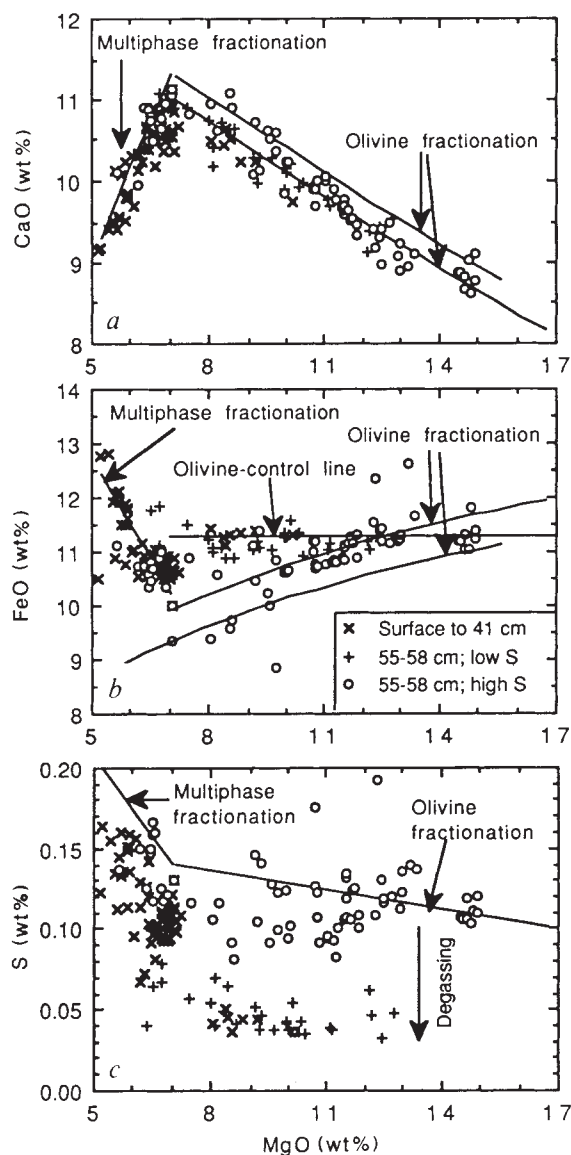


FIG. 1 Diagrams of a, CaO, b, FeO and c, S against MgO showing the high-MgO contents of many of the analysed glasses. Glasses with MgO > 7 wt% plot along olivine fractionation lines on the CaO–MgO plot. High-S glasses with MgO > 7 wt% also generally plot along olivine fractionation curves on the FeO–MgO plot, but the low-S glasses with MgO > 7 wt% have constant FeO and plot along an olivine control line. The olivine fractionation lines are calculated in increments of 2% crystallization with the equilibrium olivine composition recalculated for each increment. The olivine control line shown is for accumulation of olivine with an average composition Fo<sub>88.1</sub>. Glasses with MgO < 7 wt% plot along multiphase fractionation lines on both the CaO–MgO and the FeO–MgO plots. On the S–MgO plot, the pillow rim glasses<sup>6</sup> contain less S than the model fractionation trends shown. High-S glasses are degassed relatively little, whereas other glasses have undergone degassing of S and H<sub>2</sub>O.



TABLE 1 Microprobe analyses of glass and olivine, and an average calculated normalized composition for Kilauea primary tholeiitic melt

| Sample                         | 58-7V<br>high-Ti<br>glass | Average*<br>of 7 glasses<br>55-58 cm | 57-G7D<br>glass | 57-G14B<br>glass | 57-15D<br>glass | 57-15B<br>olivine | 57-13G<br>olivine | Primary<br>melt |
|--------------------------------|---------------------------|--------------------------------------|-----------------|------------------|-----------------|-------------------|-------------------|-----------------|
| SiO <sub>2</sub>               | 49.3                      | 48.81                                | 49.0            | 50.4             | 49.5            | 40.7              | 41.4              | 48.5            |
| Al <sub>2</sub> O <sub>3</sub> | 13.7                      | 10.99                                | 11.4            | 12.4             | 12.0            | —                 | —                 | 10.2            |
| Fe <sub>2</sub> O <sub>3</sub> | —                         | —                                    | —               | —                | —               | —                 | —                 | 1.29            |
| FeO                            | 11.7                      | 11.24                                | 11.2            | 10.8             | 11.2            | 8.95              | 9.01              | 10.4            |
| MnO                            | —                         | —                                    | —               | —                | —               | 0.13              | 0.13              | 0.17            |
| MgO                            | 7.65                      | 14.72                                | 13.0            | 11.0             | 12.5            | 49.2              | 49.2              | 17.1            |
| NiO                            | —                         | 0.070                                | —               | —                | —               | 0.55              | 0.55              | 0.086           |
| CaO                            | 10.9                      | 8.86                                 | 9.53            | 10.0             | 9.44            | 0.20              | 0.22              | 8.20            |
| Na <sub>2</sub> O              | 2.30                      | 1.70                                 | 1.82            | 1.86             | 1.77            | —                 | —                 | 1.51            |
| K <sub>2</sub> O               | 0.56                      | 0.32                                 | 0.33            | 0.36             | 0.33            | —                 | —                 | 0.270           |
| P <sub>2</sub> O <sub>5</sub>  | 0.30                      | 0.19                                 | 0.19            | 0.20             | 0.21            | —                 | —                 | 0.164           |
| TiO <sub>2</sub>               | 2.87                      | 1.95                                 | 2.06            | 2.25             | 2.09            | —                 | —                 | 1.76            |
| S                              | 0.045                     | 0.109                                | 0.122           | 0.095            | 0.033           | —                 | —                 | 0.100           |
| Cl                             | —                         | 0.011                                | —               | —                | —               | —                 | —                 | 0.010           |
| H <sub>2</sub> O               | —                         | 0.389                                | 0.421           | 0.392            | 0.228           | —                 | —                 | 0.35            |
| CO <sub>2</sub> (p.p.m.)       | —                         | 79                                   | 75              | 107              | <20             | —                 | —                 | —               |
| Total                          | 99.2                      | 99.3                                 | 99.0            | 99.8             | 99.3            | 99.8              | 100.6             | 100.0           |
| Mg#                            | 56.4                      | 72.3                                 | 69.7            | 66.9             | 68.8            | 90.7              | 90.7              | 74.5            |

Mg # is 100 Mg/(Mg + 0.9Fe) for glass and 100 Mg/(Mg + Fe) for olivine. H<sub>2</sub>O and CO<sub>2</sub> determined by Fourier transform transmission infrared spectroscopy on doubly polished glass chips.

\* NiO, H<sub>2</sub>O, CO<sub>2</sub> and Cl are averages of 2, 4, 3 and 4 analyses on separate grains, respectively.

good agreement with the NiO content determined by electron microprobe for several of the high-MgO glasses (Table 1). The high NiO contents of these olivines and their equilibrium melts may indicate that the source for Hawaiian tholeiitic basalt is enriched in NiO compared with the source beneath mid-ocean ridges, or that  $D_{Ni}$  may be lower<sup>17</sup> at the pressure where melt is segregated beneath Hawaii. To explain the data requires  $D_{Ni} = 4.4$  at the depth of melt segregation compared with  $D_{Ni} = 6.4$  at low pressure.

Viscosities<sup>18</sup> estimated for the near-primary high-MgO glass compositions and the calculated primary melt composition are only 5–3 poise, so it is unlikely that abundant olivine can be suspended to create olivine-rich magmas unless the magma is vigorously convecting. Olivine that settles into these melts from above or crystallizes from them should rapidly be deposited at the base of the magma reservoir, where it forms dunite. Dunite formed this way is commonly disrupted and sampled by subsequent postshield and rejuvenated-stage alkalic magmas.

Densities<sup>19</sup> estimated for the highest-MgO glasses and the estimated primary melt composition (both  $\sim 2.67 \text{ g cm}^{-3}$ ) are greater than for lower-MgO pillow rind glasses (as low as  $2.61 \text{ g cm}^{-3}$ ). The magma reservoir should therefore be stratified,

with hot primary and near-primary magmas ponded at the base and cooler, more-fractionated magmas overlying them. These high-MgO melts are only rarely erupted because of the presence of overlying more-fractionated melts in the magma reservoir.

About two-thirds of the glasses with MgO > 9 wt% have high S contents (>0.1 wt%), whereas the rest commonly contain <0.06 wt% S (Fig. 1c). The low-S group extends to lower MgO contents and includes the pillow rind glasses<sup>6</sup> and most glass sands with <7% MgO. Many variables control the concentration of S in melts, but variable K<sub>2</sub>O/S and K<sub>2</sub>O/H<sub>2</sub>O ratios in the pillow lavas indicate that these lavas degassed some H<sub>2</sub>O and S at low pressure near the top of the magma chamber or during subaerial eruption of part of the lava and recycling back into the magma chamber<sup>6</sup>. Volatile loss from low-density fractionated magmas increases their density to values as high as that of primary magma. Thus, degassing at the top of the magma reservoir should lead to instability, with dense, degassed, fractionated magmas at the top of the reservoir sinking to deeper levels in the reservoir where they can mix with newly introduced melts that have not been degassed, to form magmas having intermediate contents of H<sub>2</sub>O and S, like the pillow lavas from the Puna Ridge<sup>6</sup>. The data presented here indicate that only some of the highest-MgO glasses are not degassed with respect to S and H<sub>2</sub>O. The S content of the calculated average primary magma composition is 1,000 p.p.m., or  $\sim 14\%$  higher than estimated from the submarine erupted pillow lavas<sup>6</sup>. Dissolved H<sub>2</sub>O concentrations in high-MgO glasses unmodified by degassing or low-temperature addition of water (J. E. Dixon *et al.*, manuscript in preparation) correlate with K<sub>2</sub>O and P<sub>2</sub>O<sub>5</sub> contents and plot along olivine fractionation lines. The H<sub>2</sub>O/K<sub>2</sub>O ratio is  $1.24 \pm 0.07$ , slightly higher than that observed for the least degassed pillow lavas from the Puna Ridge<sup>6</sup>. Projection of the H<sub>2</sub>O–MgO trend to the 17.1 wt% MgO of the calculated primary melt gives an estimated H<sub>2</sub>O content of 0.35 wt%, or  $\sim 21\%$  lower than estimated from the submarine erupted pillow lavas<sup>6</sup>. These differences highlight the importance of these high-MgO melts, which have been minimally modified by processes in the magma reservoir. The lower-MgO pillow lavas underwent a complex array of magma chamber processes<sup>6,7</sup> including crystal fractionation, magma mixing, crystal accumulation, and degassing of some S and H<sub>2</sub>O. The highest-MgO glasses have apparently only fractionated <10% olivine (plus spinel) and degassed CO<sub>2</sub>, thereby making it comparatively straightforward

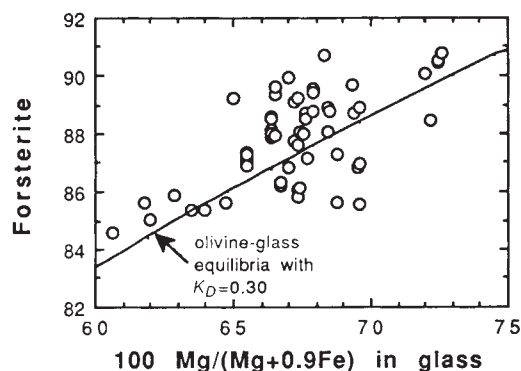


FIG. 2 Forsterite content of olivine cores plotted against  $100 \text{ Mg}/(\text{Mg} + \text{Fe}^{2+})$  of high-S glasses ( $\text{Fe}^{2+}$  is assumed to be  $0.9\text{Fe}_{\text{total}}$ ) shows that few of the olivine crystals are in equilibrium with the host glasses. The olivine-melt equilibrium line shown is for  $K_D = 0.30$ .

to extrapolate to a composition of primary Hawaiian tholeiitic melt. □

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## Herbivore-driven mycorrhizal mutualism in insect-susceptible pinyon pine

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THE mutualistic ectomycorrhizal fungi associated with the roots of woody perennials can enhance nutrient uptake and provide protection from pathogens in exchange for up to 40% of the photosynthate produced by host plants<sup>1–9</sup>. By removing photosynthetic tissue, herbivores could reduce the amount of photosynthate available for maintaining this mutualism<sup>10–15,29</sup>. Here we examine how ectomycorrhizal levels vary between trees resistant and susceptible to an insect herbivore, and demonstrate how mycorrhizal levels respond to the experimental removal of a native herbivore under natural conditions. We find that pinyon pine trees susceptible to chronic insect attack have 33% fewer ectomycorrhizae than resistant trees, demonstrating that the herbivore–mycorrhizae–host plant interaction differs between resistant and susceptible trees. We removed insects from susceptible trees and find that the mycorrhizal levels of these trees increased to a level comparable to that of resistant trees. This demonstrates that herbivores negatively affect the mutualism between ectomycorrhizal fungi and susceptible trees, and that mycorrhizal levels can rebound after herbivore removal. The dynamics of these interactions on resistant and susceptible plants could be important for understanding plant–pest interactions in natural and managed systems.

To determine whether the amount of herbivore attack was associated with the amount of mycorrhizal colonization, we examined the mycorrhizae of mature, 150-year-old pinyon pine (*Pinus edulis*) that were resistant to herbivory ( $\bar{x} \pm 1$  s.e. annual shoot mortality from 1982 to 1990 =  $8.6\% \pm 0.74$ ,  $n = 15$ ), and susceptible to herbivory ( $\bar{x} \pm$  s.e. =  $25.6\% \pm 1.60$ ,  $n = 15$ ) by a stem- and cone-boring moth, *Dioryctria albobitella*<sup>16</sup>. The larvae of this moth feed in pinyon pine stems where their chronic attack causes reduced tree growth, loss of female reproductive function, and an abnormal shrub-like architecture<sup>16</sup>. Resistant and susceptible trees grow adjacent to one another in intermixed stands and differ genetically<sup>17</sup>.

The mycorrhizal levels of resistant and susceptible trees were estimated by visually determining the percentage of short roots that were actively mycorrhizal<sup>18</sup> on a total of 80 cm of root (representing 160–200 short roots) excavated from two locations

and two sampling times for each tree. These estimates were verified by examining 10% of the roots microscopically for the presence of characteristic ectomycorrhizal structures, the Hartig net and fungal mantle<sup>19</sup>. Sampled trees grew 10–20 m apart and the complete excavation of the root systems of 10 trees demonstrated that pinyon roots from different trees rarely intermingled.

Resistant trees had significantly higher levels of mycorrhizal colonization than susceptible trees in both samples ( $F_{1,28} = 13.08$ ,  $P = 0.001$ ) (Fig. 1). The lower mycorrhizal levels of susceptible trees could be explained in three ways. First, although

TABLE 1 Levels of soil nutrients, moisture, and pH for resistant and susceptible trees

|             | NO <sub>3</sub> * N | NH <sub>4</sub> * N | PO <sub>4</sub> | % H <sub>2</sub> O | pH          |
|-------------|---------------------|---------------------|-----------------|--------------------|-------------|
| Resistant   | 6.74 ± 2.43         | 4.57 ± 0.98         | 4.94 ± 0.93     | 4.48 ± 0.30        | 7.00 ± 0.05 |
| Susceptible | 5.73 ± 1.23         | 4.61 ± 1.06         | 5.54 ± 0.99     | 4.31 ± 0.34        | 7.00 ± 0.09 |
| t-statistic | 0.203               | 0.220               | −0.443          | −0.394             | −0.045      |
| P value     | 0.841               | 0.983               | 0.663           | 0.697              | 0.964       |

Values are expressed as means  $\pm 1$  s.e. The units for soil nutrient data are  $\mu\text{g}$  nutrient per g soil. All data were analysed using a Student's *t*-test with 18 degrees of freedom<sup>27</sup>.  $n = 10$  per group.

resistant and susceptible trees are intermixed in a site, microsite differences in soil nutrients and/or moisture could affect mycorrhizae<sup>1,5</sup>. But we found no differences between resistant and susceptible trees in soil moisture, nitrate, ammonium, and phosphate, and soil pH (Table 1). Second, high herbivore loads could drive the mutualism by suppressing mycorrhizae on susceptible trees, or third, high mycorrhizal levels could directly or indirectly enhance resistance<sup>15,20</sup> such that higher mycorrhizal densities result in greater resistance to herbivory.

To help distinguish between these two hypotheses, we examined the mycorrhizal levels of susceptible trees from which the moth had been removed for a minimum of 4 years using an annual application of the systemic insecticide Cygon, and compared them with the mycorrhizal levels of resistant and susceptible control trees. Because of the difficulty of spraying large trees, a second set of mature trees, 2–4 m tall and 60 years old, were used for this experiment. Cygon reduced moth herbivory on insecticide-treated trees (1990  $\bar{x} \pm 1$  s.e. shoot mortality for susceptible treated trees =  $1.3\% \pm 0.38$ ; susceptible control trees =  $16.4\% \pm 1.82$ ; resistant trees =  $2.3\% \pm 0.69$ ,  $F_{2,42} = 57.15$ ,  $P < 0.0001$ ,  $n = 15$  per group). Soil microarthropod (for example mites, collembola) densities did not differ between resistant, susceptible, and susceptible treated trees demonstrating that Cygon had no effect on fungivores that might feed directly on mycorrhizae<sup>21–24</sup> ( $\bar{x} \pm$  s.e. soil arthropods per litre of soil for susceptible-treated trees =  $87 \pm 6.3$ ; susceptible control trees =  $78 \pm 4.5$ ; resistant trees =  $87 \pm 15.4$ , Kruskal–Wallis test statistic = 0.909,  $P = 0.635$ ,  $n = 10$  trees per group).

Susceptible trees with herbivores removed had mycorrhizal levels significantly higher than susceptible trees with herbivores, but not significantly different from resistant trees in either April or August samples ( $F_{2,42} = 25.31$ ,  $P < 0.0001$ ) (Fig. 2). This experiment demonstrates that herbivory suppresses mycorrhizae in susceptible trees and that herbivores can negatively affect this important mutualism. These data also show that the mycorrhizal differences observed between older resistant and susceptible trees also occur in the same groups of younger trees, indicating a general pattern independent of age.

Because herbivores both consume plant tissue and negatively affect an important plant–fungal mutualism, the cumulative impact of herbivory on susceptible plants is far greater than just their immediate feeding. As a result of herbivore feeding, for example, 150-year-old susceptible trees lose an average of 26% of their annual shoot production and 33% of their mycorrhizae



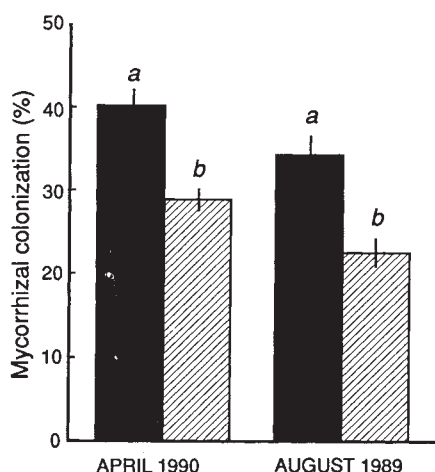


FIG. 1 Mycorrhizal levels of moth-resistant (solid bars) and moth-susceptible 150-year-old trees (hatched bars) in August 1989 and April 1990. Bars are means ( $n=15$  per group)  $\pm 1$  s.e. Bars with different letters (a, b) are significantly different from one another at  $P < 0.05$ . Data were analysed using a one-way analysis of variance with repeated measures<sup>28</sup>.

relative to resistant trees. The cumulative loss of both leaf tissue and mycorrhizae may make plants more susceptible to pathogens and other pests.

Although we have shown that herbivores mediate the mycorrhizal mutualism in susceptible trees, we do not know what drives the herbivore-plant-fungal interaction in resistant trees. The genetic differences between resistant and susceptible trees may not only lead to differential responses to herbivory through plant defenses, but also to entirely different mycorrhizal dynamics. Resistant trees, for example, could provide sufficient photosynthate to maintain mycorrhizae when they experience moderate herbivory. The improved plant nutrition resulting from mycorrhizae may then help prevent resistant plants from experiencing further insect attack. Because host-plant genetics are known to affect ectomycorrhizal formation in other pine species<sup>25</sup>, the potential for mediation of plant resistance to insect attack by mycorrhizal fungi should be considered.

Our research on the relationship between amount of herbivory

and degree of mycorrhizal colonization in pinyon pine has two general implications. First, we have shown that herbivores reduce the mycorrhizal levels of both young and old susceptible plants by an average of 33% (range 27–42%) relative to resistant trees. Because the benefits plants receive from mycorrhizae are often proportional to mycorrhizal abundance<sup>9,25,26</sup>, even small herbivore-induced mycorrhizal reductions could have significant impacts on plants, particularly those growing in stressful environments. Second, we have demonstrated that the herbivore-mycorrhizae-host plant interaction differs between resistant and susceptible trees. Because this interaction differs on insect-resistant and insect-susceptible trees, and because mycorrhizae are essential for plant growth in stressful environments<sup>9</sup>, future studies should integrate this mutualism into the body of theory dealing with plant-herbivore interactions. □

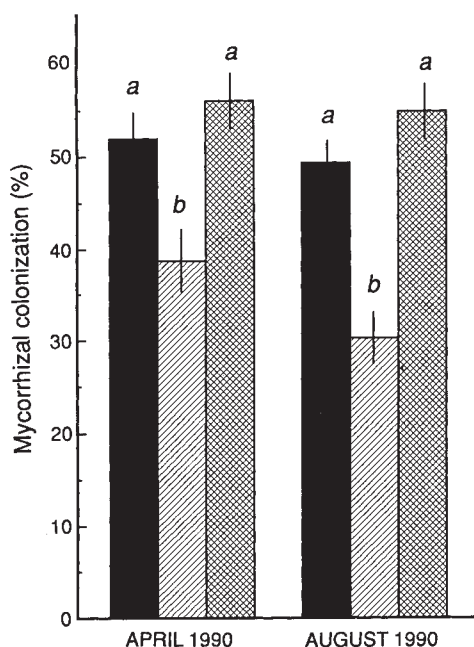


FIG. 2 Mycorrhizal levels of moth-resistant (solid bars), moth-susceptible (hatched bars), and susceptible moth-removal 60-year-old trees (cross-hatched bars) in April and August 1990. Bars are means ( $n=15$  per group)  $\pm 1$  s.e. Bars with different letters (a, b) are significantly different from one another at  $P < 0.05$ . Data were analysed using a one-way analysis of variance with repeated measures followed by *a priori* comparisons to determine significant differences between treatments<sup>28</sup>.

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# Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors

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**LONG-TERM** potentiation (LTP) in the hippocampus is thought to contribute to memory formation. In the CA1 region, LTP requires the NMDA (*N*-methyl-D-aspartate) receptor-dependent influx of  $\text{Ca}^{2+}$  and activation of serine and threonine protein kinases. Because of the high amount of protein tyrosine kinases in hippocampus and cerebellum<sup>1,2</sup>, two regions implicated in learning and memory, we examined the possible additional requirement of tyrosine kinase activity in LTP. We first examined the specificity in brain of five inhibitors of tyrosine kinase<sup>3-5</sup> (Table 1) and found that two of them, lavendustin A and genistein, showed substantially greater specificity for tyrosine kinase from hippocampus<sup>6</sup> than for three serine-threonine kinases: protein kinase A, protein kinase C, and  $\text{Ca}^{2+}$ /calmodulin kinase II. Lavendustin A and genistein selectively blocked the induction of LTP when applied in the bath or injected into the postsynaptic cell. By contrast, the inhibitors had no effect on the established LTP, on normal synaptic transmission, or on the neurotransmitter actions attributable to the actions of protein kinase A or protein kinase C. These data suggest that tyrosine kinase activity could be required postsynaptically for long-term synaptic plasticity in the hippocampus. As  $\text{Ca}^{2+}$ /calmodulin kinase II or protein kinase C seem also to be required<sup>7,8</sup>, the tyrosine kinases could participate postsynaptically in a kinase network together with serine and threonine kinases.

High-frequency stimulation of the Schaffer collateral/commissural fibre pathway to the CA1 pyramidal cells leads to three sequential and well-characterized changes in synaptic effectiveness: (1) post-tetanic potentiation lasting 2 min; (2) decremental facilitation lasting 30–40 min; and (3) persistent LTP lasting hours<sup>13</sup>. To examine the functional consequences of inhibiting tyrosine kinase activity on synaptic transmission and on these three forms of synaptic plasticity, we bathed hippocampal slices

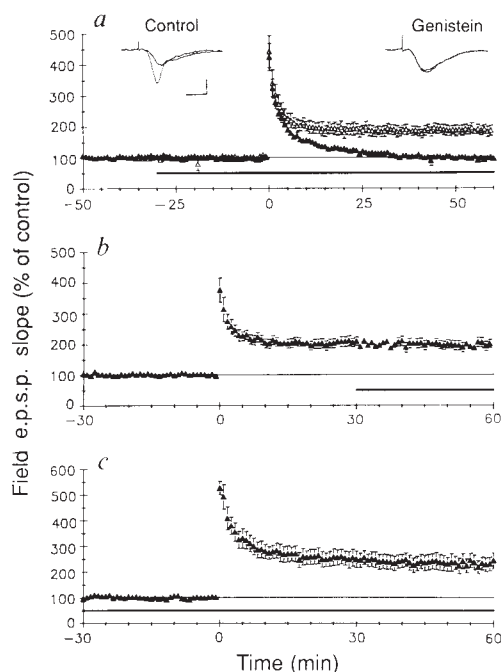
in tyrosine kinase inhibitors and evoked LTP by stimulating the Schaffer collateral pathway. Genistein (Fig. 1*a*) and lavendustin A (Fig. 2*a*) had no significant effect on control synaptic transmission, on post-tetanic potentiation or on the initial decremental facilitation, but selectively blocked LTP. Lavendustin A was more potent than genistein. Concentrations as low as 5.0  $\mu\text{M}$  completely blocked the induction of LTP when applied before the tetanus (Fig. 2*a*). This is consistent with our *in vitro* kinase assays showing lavendustin A was more potent than genistein in inhibiting pp60<sup>c-src</sup>(+) activity, a tyrosine kinase enriched in the CA1 region<sup>6</sup> (Table 1). This blockade was selective for induction; neither genistein nor lavendustin A affected LTP once it was established (Fig. 1*b*, Fig. 2 legend). Other tyrosine kinase inhibitors (Compound 5 and tyrphostin) also blocked the induction of LTP, but in the case of tyrphostin, the block was less complete.

The data suggest that tyrosine kinases are required for the induction of LTP. We next attempted to determine whether the relevant tyrosine kinases required for induction are located in the presynaptic terminals or in the postsynaptic CA1 pyramidal cells. We injected lavendustin A into the postsynaptic cells 30 min before the tetanus and again blocked the induction of LTP (Fig. 2*b*). By contrast, intracellular lavendustin A had no effect on normal synaptic transmission (Fig. 2 legend). These findings suggest that the induction of LTP requires tyrosine kinase activity in the postsynaptic pyramidal cells.

To examine further the physiological specificity of these tyrosine kinase inhibitors, we did additional control experiments. First, we examined genistin, a structural analogue of genistein that does not block tyrosine kinase activity, and found that it failed to block LTP when applied extracellularly before high-frequency stimulation (Fig. 1*c*).

Second, we examined the effects of these inhibitors on the AMPA ( $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and NMDA receptor-mediated components of the synaptic potentials produced by activation of the Schaffer collaterals. The inhibitors had no effect on the AMPA receptor component, which contributes predominantly to normal synaptic transmission (Fig. 1). Similarly, lavendustin A had no effect on the NMDA receptor-mediated synaptic component produced in presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10  $\mu\text{M}$ ) that blocks the AMPA receptor (Fig. 3*a*).

Finally, we tested the specificity of tyrosine kinase inhibitors



**FIG. 1** *a*, Bath application of genistein (110  $\mu\text{M}$ , 0.33% DMSO) before tetanic stimulation blocks LTP ( $N=7$ ). Each point in this and subsequent figures represents the mean  $\pm$  s.e.m. Traces are responses evoked 5 min before and 60 min after tetanic stimulation. Vehicle alone (0.33% DMSO) does not block LTP (filled triangles,  $N=5$ ). Calibration bars: 1 mV; 6.5 ms. *b*, Genistein (110  $\mu\text{M}$ ) applied 30 min after tetanic stimulation does not block LTP ( $N=7$ ). No effect of genistein applied 30 min post-tetanus was observed in three of these slices where genistein was applied for at least 55 min. *c*, An analogue of genistein that lacks anti-tyrosine kinase activity, genistin (110  $\mu\text{M}$ , 0.33% DMSO), does not inhibit LTP when applied for at least 30 min pretetanus ( $N=7$ ).

**METHODS.** Transverse slices 400  $\mu\text{M}$ -thick of guinea pig hippocampus were maintained in an interface chamber at 30  $^{\circ}\text{C}$  and subfused (1–3  $\text{mL min}^{-1}$ ) with a saline consisting of (in mM): 124.0 NaCl, 4.4 KCl, 25.0  $\text{NaHCO}_3$ , 1.0  $\text{Na}_2\text{H}_2\text{PO}_4$ , 2.0  $\text{CaCl}_2$ , 2.0  $\text{MgSO}_4$ , and 10.0 glucose. A bipolar, platinum-iridium stimulating electrode was placed in the stratum radiatum of the CA1 region and extracellular field potentials were recorded using a microelectrode (5–8 Mohm, 2.5 M NaCl) also placed in the stratum radiatum. The stimulation intensity was adjusted to give field e.p.s.p. amplitudes of about 1.0 mV and responses were elicited at 0.02 Hz. LTP was induced by two 1.0-s long trains of 100-Hz stimulation (intertrain interval=20 s). The first response post-tetanus was elicited 8.0 s after the last high-frequency train. Drugs were applied either in the bath or by drop application of drug-containing saline onto the slice from a glass micropipette positioned near the recording electrode.



TABLE 1 Specificity of tyrosine kinase inhibitors on kinase substrate phosphorylation

|                  | src   | PKC   | PKA    | CamKII |
|------------------|-------|-------|--------|--------|
| Genistein        | 18    | 185   | >200   | >200*  |
| Genistin         | >200* | >200* | >200*  | >200*  |
| Lavendustin A    | 0.5   | >100* | >100*  | >100*  |
| Compound 5       | 0.5   | 70    | >100** | 0.2    |
| Trypstin RG50864 | 8     | 60    | >100   | 65     |

Inhibitory concentration ( $IC_{50}$ ) values ( $\mu M$ ) determined by *in vitro* kinase assays. Genistein (ICN) was at least 10-fold more potent in inhibiting  $pp60^{c-src(+)}$  than it was in inhibiting PKC, CamKII or PKA. By contrast, the inactive genistein analogue, genistin (Extrasynthese), did not inhibit these kinases. Lavendustin A (Gibco BRL) was more potent than genistein on  $pp60^{c-src(+)}$  and showed a wider range of specificity, being at least 200-fold less effective at inhibiting PKC, CamKII and PKA. Compound 5 (2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid; Gibco BRL); an analogue of lavendustin A, was also effective in inhibiting  $pp60^{c-src(+)}$  but was not specific; it potentially inhibited CamKII. Trypstin RG50864, a substrate competitor, was less specific than the ATP competitors genistein or lavendustin A. In addition to inhibiting  $pp60^{c-src(+)}$ , a cytoplasmic tyrosine kinase, all of these inhibitors potentially inhibit receptor tyrosine kinases<sup>3-5</sup>. The  $pp60^{c-src}$  was immunoprecipitated from hippocampus extracts using monoclonal antibody 327 (ref. 24; Oncogene Science) and used to phosphorylate poly (Glu, Tyr) 4:1 (ref. 25; Sigma). Rat brain purified PKC (Lipidex) was used to phosphorylate [Ser]-PKC (19-31) peptide<sup>25</sup> (Peninsula laboratories) in the presence of  $Ca^{2+}$ , phosphatidyl serine and TPA as described by Kikkawa *et al.*<sup>26</sup>. Heart PKA catalytic subunit (Sigma) phosphorylation of kemptide (Peninsula Laboratories) was done as described by Roskoski<sup>27</sup>. Rat brain  $Ca^{2+}$ /calmodulin-dependent CamKII phosphorylation of syntide-2 (gift of R. J. Colbran) was done as described by Hashimoto and Soderling<sup>28</sup>. All assays had a final concentration of 2% DMSO to dissolve inhibitors and ATP was 10  $\mu M$ , and values are representative of duplicate or more experiments.

\* No inhibition of kinase activity was seen at this concentration.

by examining their effects on transmitter actions thought to be mediated by the intracellular activation of two serine-threonine kinases, protein kinase C (PKC) and protein kinase A (PKA). Muscarinic agonists that elicit the production of diacylglycerol and inositol trisphosphate inhibit the slow afterhyperpolarization in hippocampal neurons<sup>9</sup>. This inhibition is mimicked by phorbol esters and is thought to be mediated partly by PKC (ref. 10). Concentrations of genistein or lavendustin A that blocked LTP had no effect on carbachol inhibition of the slow

after hyperpolarization when delivered either extra- or intracellularly (Fig. 3c). As an additional test of the selectivity of these inhibitors to tyrosine kinases compared with PKC activity, we examined the effects of genistein on the potentiation of synaptic transmission produced by directly activating PKC with phorbol esters<sup>11</sup>. This facilitation was partially suppressed by the general kinase inhibitor H-7 (300  $\mu M$ ), yet concentrations of genistein that block LTP had no effect on the phorbol ester-induced potentiation (Fig. 3d), providing further evidence that this concentration of genistein has little effect on PKC activity. An inhibition of the afterhyperpolarization, similar to that produced by carbachol, is also achieved by  $\beta$  adrenergic receptor agonists through an increase in cAMP and presumably PKA (ref. 12). This inhibition was also not inhibited by genistein (Fig. 3c). These physiological experiments, together with the biochemical experiments of Table 1, suggest that the concentrations of genistein and lavendustin A that block LTP do not affect the serine-threonine kinases (PKC, PKA and  $Ca^{2+}$ /calmodulin kinase II (CamKII)). Although we cannot exclude an effect on other kinases, the blockade of LTP is most readily explained by inhibition of tyrosine kinases.

How might tyrosine kinase activity contribute to the induction of LTP? The initial steps in the induction of LTP in the CA1 region of the hippocampus are thought to involve  $Ca^{2+}$  influx through NMDA receptors and the subsequent activation in the postsynaptic cell of one or both of the kinases PKC and CamKII (refs 7, 8, 13). Our data suggest that in addition to these serine-threonine kinases the induction of LTP requires tyrosine kinase activity.

There are at least four ways in which tyrosine kinases could be activated so as to contribute to a network of kinase activity in the postsynaptic cell. First, a tyrosine kinase could be activated directly after tetanic stimulation: for example, the  $Ca^{2+}$  influx mediated by the NMDA receptor might directly trigger tyrosine kinase activity. Indeed, a  $Ca^{2+}$ /calmodulin-dependent tyrosine kinase activity has been described<sup>14,15</sup>. Second, the tyrosine kinase might be activated indirectly by PKC or CamKII in a series cascade of kinase activation (see, for example, ref. 16). Third, a downstream kinase, such as MAP kinase<sup>17</sup>, may act as a convergence point for parallel serine and tyrosine phosphorylation. Finally, as our data on tyrosine kinases (and almost all the existing data on the serine-threonine kinases) are based on the use of kinase inhibitors, these kinases may not be activated by tetanus. To initiate LTP, constitutive activity of tyrosine

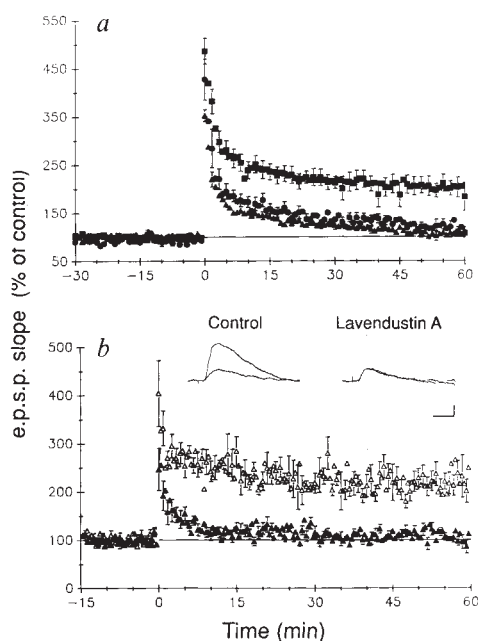
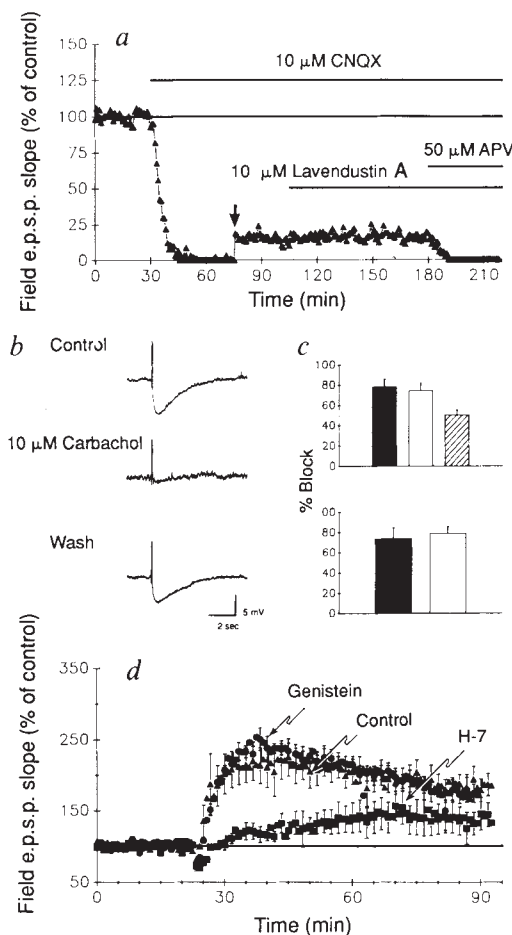


FIG. 2 a, Lavendustin A blocks LTP. Ten  $\mu M$  ( $\blacktriangle$ ), 5  $\mu M$  ( $\bullet$ ), and 0  $\mu M$  ( $\blacksquare$ ) lavendustin A were applied for 0.5–4.0 h in saline containing 0.1% DMSO and 25  $\mu M$  ascorbate before tetanic stimulation ( $N \geq 5$ ). Lavendustin A (10  $\mu M$ ) applied for 1 h beginning 30 min post-tetanus had no effect on established LTP ( $N = 3$ , data not shown). b, Postsynaptic injection of lavendustin A blocks LTP.  $\triangle$ , control experiments ( $N = 8$ ). In experiments with 1.0 mM lavendustin A in the intracellular electrode ( $N = 8$ ), LTP of the intracellularly recorded e.p.s.p.s was blocked ( $\blacktriangle$ ). The responses shown were elicited 5 min pretetanus and 1 h post-tetanus. Calibration bars: 5 mV; 18.5 ms.

METHODS. Intracellular recordings were obtained with microelectrodes filled with 3.0 M CsCl (resistance, 40–80 M $\Omega$ ) containing either 0.5% DMSO or 0.5% DMSO plus 1.0 mM lavendustin A. The e.p.s.p.s (stimulation rate, 0.04 Hz) were monitored for at least 15 min before high-frequency stimulation. Tetanic stimulation was delivered at least 30 min after penetration of a cell with the microelectrode to allow diffusion of lavendustin A into the cell. Data included in the averages are from experiments where field e.p.s.p.s recorded by a second, extracellular electrode, were at least 130% of control 1 h post-tetanus. In these experiments the CA3 region was removed and the saline contained 20  $\mu M$  picrotoxin and 4.0 mM  $CaCl_2$  and  $MgSO_4$ . Intracellular delivery of lavendustin A had no effect on normal synaptic transmission in experiments with 1.0 mM lavendustin A containing intracellular microelectrodes where no tetanus was applied at 30 min poststimulation and e.p.s.p.s were monitored over the next 60 min. (e.p.s.p.s at the end of these experiments were  $98.4 \pm 11.8\%$  (mean  $\pm$  s.e.m.  $N = 5$ ) of those recorded during the first 15 min.)

FIG. 3 *a*, Lavendustin A (10  $\mu$ M) does not block NMDA receptor-mediated field e.p.s.p.s. Bath application of the non-NMDA receptor antagonist CNQX (10  $\mu$ M, 0.01% DMSO) lead to a near complete block of the field e.p.s.p.s. Increasing the stimulation strength (arrow) resulted in a small NMDA receptor-mediated response that was not inhibited by lavendustin A but was fully blocked by 50  $\mu$ M 2-amino-5-phosphonopivalic acid. NMDA receptor-mediated e.p.s.p.s following a 1-h application of lavendustin A (10  $\mu$ M) were  $116 \pm 0.03\%$  (mean  $\pm$  s.e.m.;  $N=5$ ) of prelavendustin A amplitudes. *b*, Representative traces showing the slow afterhyperpolarization elicited by a 50 ms depolarizing current pulse delivered through the recording electrode before (top), after drop application of 10  $\mu$ M carbachol (middle), and after carbachol washout (bottom). *c*, Summary of the ability of carbachol (top) and norepinephrine (bottom) to inhibit the slow after hyperpolarization in the presence of protein kinase inhibitors. Genistein (110  $\mu$ M; open bars) did not prevent the action of carbachol ( $N=9$ ) or norepinephrine ( $n=5$ ) on the slow afterhyperpolarization ( $P>0.05$ ). High concentrations of the PKC inhibitor H-7 (300  $\mu$ M; hatched bars) produced a small (25%,  $N=7$ ) inhibition of the action of carbachol. The ability of carbachol to block the slow afterhyperpolarization was not affected in experiments where lavendustin A (1.0 mM, 0.5% DMSO) was present in the electrode solution (3.0 M K-acetate) and allowed to diffuse into the cells for 30–90 min ( $N=8$ , data not shown). *d*, Separate groups of slices were used as controls ( $N=9$ ) or preincubated for 1–4 h in genistein (110  $\mu$ M, 0.33% DMSO,  $N=6$ ) or H-7 (300  $\mu$ M, 0.2% DMSO,  $N=8$ ). Drop application of 10  $\mu$ M phorbol 12, 13-diacetate (PDAC)-containing saline onto the slice resulted in a rapid potentiation of synaptic transmission ( $\blacktriangle$ ) that was not inhibited by genistein ( $\bullet$ ). H-7 (300  $\mu$ M) ( $\blacksquare$ ) suppressed the response to PDAC; the PDAC-induced potentiation was significantly less ( $P<0.01$ ) in the presence of H-7 only for the first 30 min after PDAC application.

**METHODS.** Intracellular recordings were obtained from CA1 pyramidal cells using 40–80 Mohm microelectrodes filled with 3.0 M K-acetate or 2.5 M K-methylsulphate (pH 7.4). Following a stable impalement, brief (50–60 ms), strongly depolarizing current pulses were delivered through the recording electrode to elicit the slow afterhyperpolarization. After measuring three to five control responses, drops of either 10  $\mu$ M carbachol or 10  $\mu$ M norepinephrine (plus 10  $\mu$ M ascorbate) dissolved in saline were applied to the exposed surface of the slice. Current was delivered through the recording electrode to return the membrane potential to control values (typically  $-55$  to  $-65$  mV) before eliciting the slow afterhyperpolarization in the presence of agonist. Kinase inhibitors were applied in the bath saline for at least 30 min before agonist application.



kinases may simply be required in the postsynaptic cell in conjunction with NMDA receptor activation.

Although LTP is induced postsynaptically, the maintenance of LTP seems to involve enhanced transmitter release from the presynaptic neuron<sup>19,20</sup>. This suggests that postsynaptic induction leads to the release of a retrograde signal, perhaps a cell-permeable metabolite such as arachidonic acid or nitric oxide (NO), which then diffuses from the postsynaptic to the presynaptic cell<sup>18–20</sup>. Thus, one possibility suggested by our findings is that the enzymes involved in generating the retrograde signal either require phosphorylation by both tyrosine and serine-threonine kinases or are phosphorylated by an enzyme such as MAP kinase that is itself a convergence point for both kinases.

It is therefore interesting that nitric oxide synthase, the enzyme that generates NO, is highly regulated. It is a substrate for all three major serine-threonine kinases (CamKII, PKC and PKA) and has, in addition, putative phosphorylation sites for tyrosine kinase (see ref. 21 and D. S. Bredt *et al.*, manuscript submitted). Following up on this possibility we have found that injection into the postsynaptic cell of specific inhibitors of NO synthase block the induction of LTP and application of NO enhances spontaneous release from hippocampal neurons in culture<sup>29</sup>.

Finally, in addition to being induced by tetanic stimulation of the Schaffer collaterals, LTP in CA1 can be modulated by other factors, such as EGF and FGF<sup>22,23</sup>, that act through tyrosine kinase receptors. □

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# The *int-2* proto-oncogene is responsible for induction of the inner ear

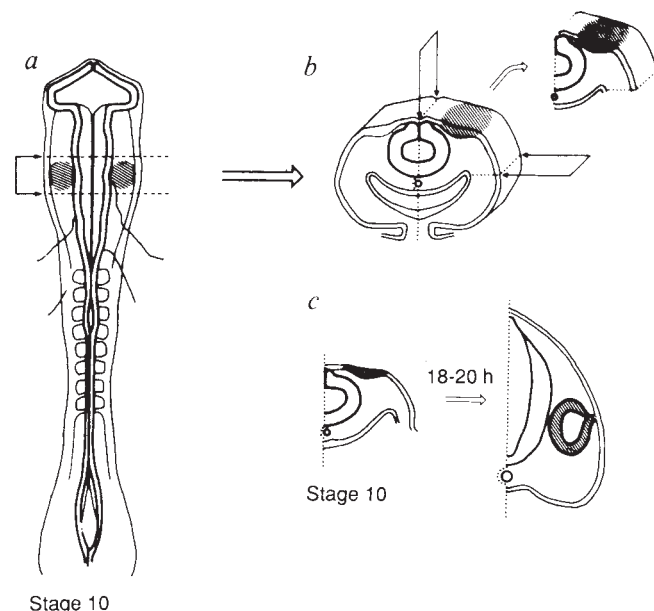
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THE *int-2* proto-oncogene encodes several products related to the fibroblast growth factor (FGF) family<sup>1,2</sup>. FGFs have been associated with mesoderm induction in the amphibian embryo<sup>2,3</sup> and *int-2* has a distinct pattern of expression throughout development in vertebrates<sup>4,5</sup>. But evidence for a function of *int-2* in embryogenesis has been lacking. In the mouse embryo, *int-2* transcripts have been detected in the rhombencephalon at a developmental stage where classical experiments showed that the induction of the inner ear occurs<sup>6,7</sup>. This raises the possibility that *int-2* may constitute a signal for the induction of the otic vesicle, the primordium of the inner ear. We provide direct evidence for this view by showing that (1) the formation of the otic vesicle is inhibited by antisense oligonucleotides targeted to the secreted form of *int-2*, and by antibodies against *int-2* oncoproteins, and (2) basic FGF (bFGF) can mimic the inductive signal in the absence of the rhombencephalon.

The induction of the inner ear requires two overlapping inductive waves<sup>6,7</sup>. The first originates from the chorda mesoderm, stages 7 to 9, and specifies the ectodermal field that will become the otic placode. The second arises from the rhombencephalic neural tube, stages 9 to 17, and induces the otic vesicle<sup>8</sup>. It is during these stages that expression of *int-2* has been detected in the rhombencephalon<sup>5</sup>. We did experiments to reproduce the formation of the otic vesicle *in vitro*. Chick embryo explants containing the rhombencephalon (rh(+)) and the ectodermal presumptive area were grown in culture and the development of the otic vesicle followed (Fig. 1). When explants were grown with 10% serum, the formation of the otic vesicle was completed in about 18–20 h (Figs 1c and 2a). This was prevented by removal of the rhombencephalic neural tube (rh(-)). Therefore, the normal rhombencephalic induction was reproduced *in vitro* with similar time scale and requirements as observed *in vivo*.



Stage 10

TABLE 1 Effect of *int-2* blockade on otic vesicle formation

|                                             | $V_t$<br>( $\times 10^{-7}$<br>$\text{cm}^3$ ) | Cell<br>number<br>( $\times 10^3$<br>per<br>vesicle) | Mitosis<br>(per<br>vesicle) | $\alpha$<br>( $\times \pi$<br>radians) | Stage |
|---------------------------------------------|------------------------------------------------|------------------------------------------------------|-----------------------------|----------------------------------------|-------|
| rh(+)                                       | 33.0 $\pm$ 1.1                                 | 26.4 $\pm$ 0.9                                       | 220 $\pm$ 24                | 2.0 $\pm$ 0.03                         | 16–17 |
| rh(+)<br>+AUG- <i>int-2</i> antisense       | 7.7 $\pm$ 0.3                                  | 6.2 $\pm$ 0.2                                        | 22 $\pm$ 2                  | 0.3 $\pm$ 0.04                         | 10    |
| rh(+)<br>+AUG- $\beta$ -globin<br>antisense | 29.2 $\pm$ 1.0                                 | 23.4 $\pm$ 0.8                                       | 219 $\pm$ 10                | 2.0 $\pm$ 0.02                         | 16–17 |
| rh(+)<br>+random oligo-1                    | 28.3 $\pm$ 2.0                                 | 23.3 $\pm$ 1.7                                       | 209 $\pm$ 12                | 2.0 $\pm$ 0.01                         | 16–17 |
| +random oligo-2                             | 27.6 $\pm$ 2.6                                 | 21.2 $\pm$ 2.0                                       | 227 $\pm$ 13                | 1.9 $\pm$ 0.02                         | 15–17 |
| rh(+)<br>+ $\alpha$ - <i>int-2</i> antibody | 11.2 $\pm$ 0.7                                 | 9.0 $\pm$ 0.6                                        | 51 $\pm$ 5                  | 0.4 $\pm$ 0.1                          | 11–12 |
| rh(-)<br>+bFGF                              | 8.7 $\pm$ 0.2                                  | 7.0 $\pm$ 0.2                                        | 33 $\pm$ 4                  | 0.2 $\pm$ 0.05                         | 10    |
| rh(-)<br>+EGF+I                             | 26.5 $\pm$ 0.8                                 | 21.2 $\pm$ 0.6                                       | 173 $\pm$ 7                 | 1.8 $\pm$ 0.1                          | 16    |
|                                             | 14.1 $\pm$ 1.8                                 | 11.3 $\pm$ 1.4                                       | 77 $\pm$ 11                 | 0.2 $\pm$ 0.1                          | 10    |

rh(+), Explants containing the rhombencephalic neural tube; rh(-), those in which it was removed. The volume of vesicular tissue,  $V_t$ , cell number and mitotic number were measured as described in ref. 13.  $\alpha$  is the associate central angle of an ideal arc inscribed in the central section of the vesicle and gives a measure of the degree of invagination without reference to the absolute mass of tissue. The column labelled stage indicates the equivalent developmental stage (ref. 15) to which the cultured otic vesicle would correspond *in vivo*. Epidermal growth factor and insulin (EGF+I) were 20 nM and 1  $\mu\text{g ml}^{-1}$ , respectively. Random oligonucleotides contained the same base composition as the (AUG)*int-2* antisense but in a different order. Sequences were CTCACAGACACCGGT for random oligo-1 and AACGCACTCAGCTGC for random oligo-2. (AUG)*int-2* antisense and anti-*int-2*-antibody had no effect on cell proliferation in either HL60 cells (48 h) or trunk neural crest cells (24 h).

Incubation of rh(+)-explants with 10% fetal calf serum (FCS) in the presence of an oligodeoxynucleotide complementary to the AUG initiation site of the human *int-2* gene, inhibited induction of the otic vesicle (Fig. 2b). The antisense oligonucleotide was not effective when targeted against the CUG upstream to the AUG codon, reported to be an alternative initiation site for the transcription of a nonsecreted product of *int-2* (ref. 9). A  $\beta$ -globin antisense oligonucleotide was used as a control in these experiments (Fig. 2c). Two other oligonucleotides containing the same base composition as the (AUG)*int-2* antisense but ordered at random were unable to inhibit induction (see below and Table 1), excluding a nonspecific effect of the oligodeoxynucleotide. Possible toxicity of oligonucleotides was also assessed by estimating the rate of cell death. Cell deaths per

FIG. 1 Induction of the otic vesicle *in vitro*. Explants were prepared from transverse sections of stage 10 chick embryos (a). They were microdissected through the planes indicated in b, to leave the rhombencephalon and the presumptive ectoderm of the otic primordium (shaded area). Explants cultured in the presence of 10% serum, developed otic vesicles in 18–20 h that corresponded to stage 17 embryos and were comparable to those developed *in vivo*<sup>11</sup> (c).

METHODS. Transverse sections of stage 10 chick embryos were aseptically isolated and microdissected by a longitudinal incision along the midline and a horizontal incision through the pharyngeal cavity. The explant was then formed by the rhombencephalic neural tube, the adjacent ectoderm and the pharyngeal endoderm. The notochord and the cephalic mesenchyme were always present in the explants. Structures other than the rhombencephalon have no effect on otic induction<sup>6,7</sup>. Explants were submerged in collagen gels<sup>12</sup> and incubated in M-199 with Earl's salts at 37 °C in 5% CO<sub>2</sub>, 18–20 h. Medium was supplemented with 10% FCS.

vesicle were  $33 \pm 3$  in antisense explants and  $44 \pm 3$  ( $n = 4$ ) in controls.

Incubation with an anti-*int-2* antibody also inhibited the induction of the otic vesicle (Fig. 2d). This suggests further that the *int-2* product needs to be secreted to display its inductive effect. The same antibody was used by immunoblotting to identify the naturally occurring products of *int-2* (Fig. 3a). It recognized predominantly a protein of relative molecular mass 27,000 ( $M_r$ , 27K) that corresponds to the molecular species found in the secretory pathway of COS-1 cells transfected with AUG clones of *int-2* (ref. 9). Figure 3a (middle lanes) also shows that incubation of explants with (AUG)*int-2* antisense produced the loss of the 27K band, others being unaffected. Note that the 32–33K bands also recognized by the antibody were sensitive to incubation with the (CUG)*int-2* antisense without affecting the 27K product and vice versa (two right-hand lanes). These experiments indicate first a high specificity in antisense effects and, second that although other forms are present the 27K product seems to be the one required for otic induction.

As mentioned above, otic vesicle formation did not occur in rh(-) explants, either in the presence or absence of serum.

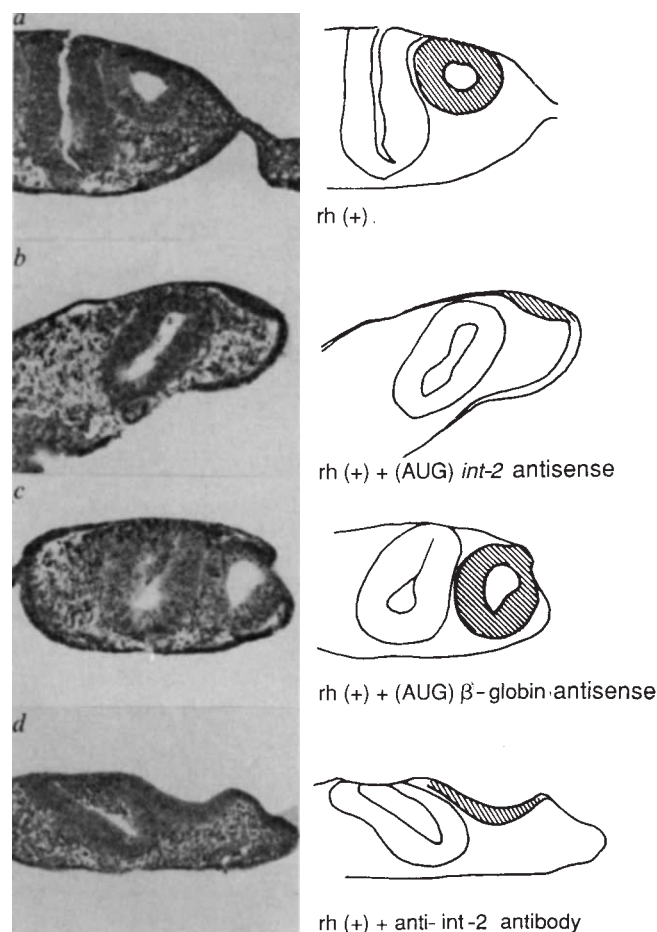


FIG. 2 Suppression of the induction of the otic vesicle by *int-2* inhibition. Photomicrographs of sections of explants containing the rhombencephalic neural tube, rh(+), and cultured with 10% serum: with no further additions (a); in the presence of  $10 \mu\text{M}$  (AUG)*int-2* antisense oligonucleotide (b); in the presence of  $10 \mu\text{M}$  (AUG) $\beta$ -globin antisense oligonucleotide (c); and in the presence of an  $\alpha$ -*int-2* antibody (1:200) (d). The diagrams are reconstructions of the sections and the shaded area represents the otic primordium. The (AUG)*int-2* antisense oligonucleotide was CCAGATCAGGCCCAT, complementary to the first 15 nucleotides starting from the ATG initiation codon<sup>1</sup> and similarly, for the (AUG) $\beta$ -globin oligonucleotide<sup>13</sup>, AGTCCAGTGCACCAT. Antibody against human *int-2* was purchased from Cambridge Research Biochemicals, UK. Histological methods are as in ref. 14.

Figure 3b shows that the addition of bFGF ( $200 \text{ ng ml}^{-1}$ ) to rh(-) cultures could replace the rhombencephalon in producing the invagination of the otic vesicle. This was also the case for rh(+) explants incubated in the presence of the (AUG)*int-2* antisense, where bFGF also rescued induction (not shown). Lower concentrations of bFGF ( $50$ – $100 \text{ ng ml}^{-1}$ ) produced incomplete recovery of the induction in rh(-) explants. Therefore, bFGF which shows structural homology with the predicted sequence of *int-2* was able to faithfully mimic the rhombencephalic inductive signal.

The blockade of *int-2* by the antisense oligonucleotide or the antibody had a strong effect on the cell proliferation rate of the otic primordium (Fig. 3c). Estimations of mitotic rate and cell number were performed under the different experimental conditions (Table 1). They revealed a reduction of the mitotic activity

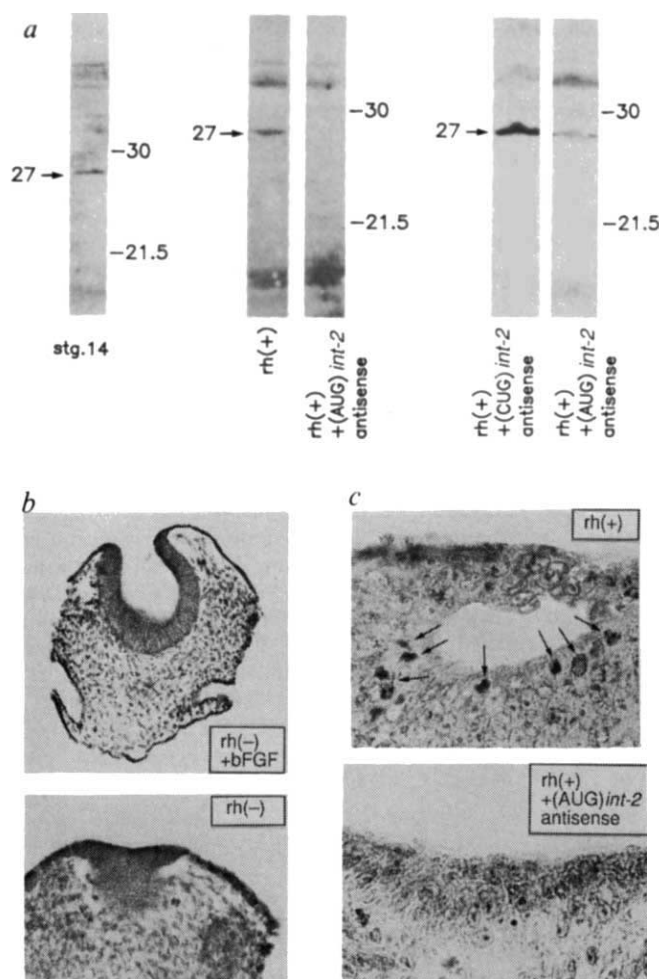


FIG. 3 a, *int-2* oncoprotein in otic explants. Lysates from explants were analysed by SDS-PAGE in a 12% gel and western blotting done using an antibody to *int-2* oncoprotein (Cambridge Research Biochemicals, UK) developed with enhanced chemoluminescence (Amersham). The numbers indicate the apparent  $M_r$  ( $\times 10^{-3}$ ). Lane labelled stg.14 corresponds to a lysate from an explant of stage 14. The others are from explants obtained at stage 10 and grown in culture for 20 h in the conditions indicated at the bottom of each lane (abbreviations as in Fig. 2). b, bFGF can replace the rhombencephalic neural tube. rh(-) explants were incubated in the presence (top) or in the absence (bottom) of  $200 \text{ ng ml}^{-1}$  of bFGF for 20 h. c, *int-2* induces cell proliferation in the otic vesicle. Sections through a 20-h explant cultured with 10% serum (top) or with 10% serum plus  $5 \mu\text{M}$  (AUG)*int-2* antisense (bottom). Note the abundance of mitotic figures (arrows) towards the luminal aspect of the otic epithelium in the rh(+) section (top) in contrast to the absence of mitotic activity in the presence of (AUG)*int-2* antisense oligonucleotide (bottom). Methods as in refs 11 and 14.



in the presence of the (AUG)*int-2* antisense oligonucleotide or *int-2* antibody with values similar to rh(-) explants. By contrast, rh(-) explants cultured in the presence of bFGF showed a proliferation rate comparable to normal. The degree of invagination ( $\alpha$ ) correlated positively with cell proliferation. Heparin ( $25 \mu\text{g ml}^{-1}$ ) which is known to bind FGF-factors<sup>3</sup>, also reduced the mitotic rate, cell number and degree of invagination by the same extent as the anti-*int-2* antibody used as at 1:200 dilution.

Other molecules that disclose mitogenic effects on the otic vesicle<sup>11</sup> were examined. Epidermal growth factor (Table 1) and bombesin (not shown) were both able to stimulate cell proliferation in rh(-) explants. But the increase in cell number and mitotic index did not correlate in this case either with the degree of invagination ( $\alpha$ ), or with the morphological arrangement of the otic placode (Table 1).

These experiments support the view that *int-2* is a natural inductive signal for the otic vesicle. This is consistent with previous work showing the transient expression of the *int-2* gene in the rhombencephalon<sup>5</sup> and the diffusible nature of the rhombencephalic inductive signal<sup>7</sup>. The requirement of *int-2* is specific as the blockade of *int-2* could not be released by other growth factors but only by addition of bFGF. In developmental stages later than those focused here, *int-2* transcripts have also been detected in the otic vesicle, in the area that will become sensory epithelium<sup>5</sup>. The development of the inner ear would then possibly fit with the 'two-stage' model of action of *int-2* proposed by Acland *et al.*<sup>9</sup>. The AUG-initiated *int-2* protein, originated in the rhombencephalic neural tube, would serve as a secreted signal for the induction and formation of the otic vesicle and the CUG-initiated oncoprotein expressed in the otic vesicle could be related to the differentiation of the sensory epithelium at more advanced development stages.

The effect of *int-2* on the otic placode was to increase the cell proliferation rate, which appears to be a necessary requirement for invagination and folding<sup>10</sup>. Besides its mitogenic effect, *int-2* most probably triggers additional mechanisms such as expression of cell adhesion molecules, basement membrane organization and secretion of extracellular matrix, which are known to contribute to the morphogenesis of embryonic epithelia, including the otic vesicle.

Sensory elements derived from ectodermal placoda include neuronal populations of cranial ganglia and the inner ear. The expression of *int-2* occurs sequentially in regions proposed as inductive areas for ectodermal placoda such as the rhombencephalon for the otic vesicle and the pharyngeal pouches for epibranchial placoda. This may suggest that *int-2* could well represent a common requirement for early steps in the development of inner ear and cranial ganglia. □

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## Concentration-dependent transcriptional activation or repression by *Krüppel* from a single binding site

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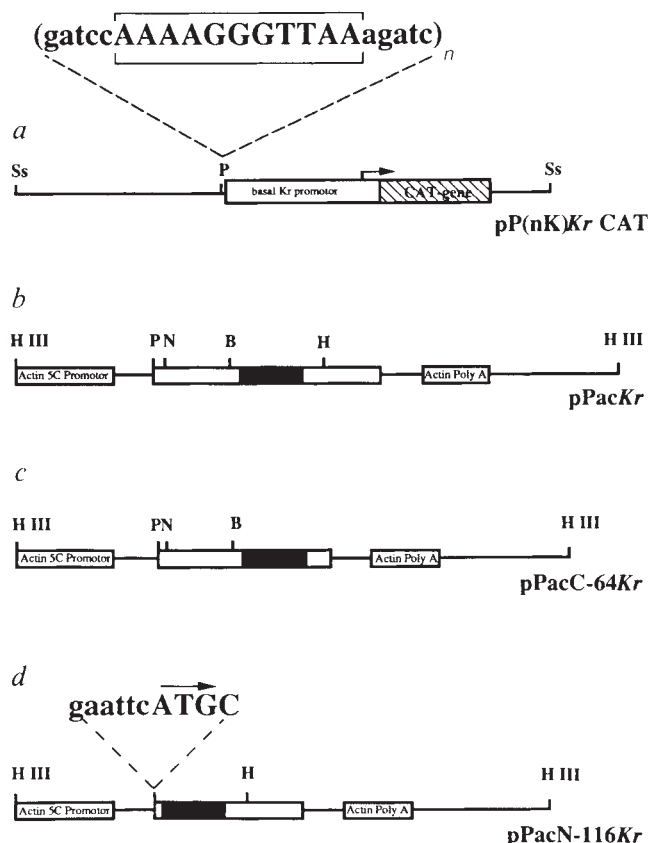
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ONE of the gap class of segmentation genes<sup>1</sup>, *Krüppel* (*Kr*), is required for normal thorax and abdominal development of the *Drosophila* embryo. Its gene product, a zinc-finger type protein<sup>2</sup>, forms a bell-shape concentration gradient in a central position of the blastoderm<sup>3-6</sup>. Genetic and molecular studies suggested that the *Kr* protein (KR) may act both as a positive and as a negative regulator of transcription on several other genes<sup>3-9</sup> of the zygotic segmentation hierarchy<sup>1</sup>. We have examined the regulatory potential of *Kr* by a series of cotransfection experiments in the *Drosophila* Schneider cell line system. Different doses of *Kr* expression plasmid were tested for their ability to drive reporter gene expression mediated by a single 11-base pair KR *in vitro* binding site common to several putative *Kr* target genes<sup>4-6,8-10</sup>. Our results show that low amounts of *Kr* expression plasmid lead to transcriptional activation, whereas high amounts result in repression. Distinct portions of KR other than the DNA-binding domain are required for gene activation and repression, suggesting that KR itself can act as a concentration-dependent positive and negative regulator of transcription.

We constructed reporter gene plasmids (Fig. 1a) containing the 11-base pair (bp) KR *in vitro* binding site AAAAGGGTTAA (K-element) which had been identified in the *cis*-acting elements

of several putative *Kr* target genes<sup>4-6,8-10</sup>. Cultured *Drosophila* Schneider cells were transfected with DNA of the reporter gene p1KKrCat containing a single K-element (Fig. 1a). When the KR expression plasmid pPacKr (Fig. 1b) was used in cotransfection experiments with the reporter gene construct, both activation and repression of gene expression were observed; low amounts lead to transcriptional activation, whereas high amounts resulted in repression (Fig. 2a, b). Similar results were obtained with reporter constructs containing five instead of one copy of the K-element (compare Figs 2a and 3a). The basal amount of gene expression was left unchanged by cotransfected *Kr* DNA when the K-element was absent or replaced in the reporter gene DNA, and no activation or repression occurs when plasmids containing the DNA encoding transcription factors other than KR were used for cotransfections. To exclude the possibility that cryptic KR binding sites in the *Kr* basal promoter may have contributed to *Kr*-dependent gene activation or repression, we replaced this promoter with the 33-bp sequence of the distal promoter of the *Drosophila* alcohol dehydrogenase gene (*Adh*)<sup>11,12</sup>. This replacement did not alter the phenomenon of dosage-dependent activation and repression of gene expression (Fig. 3b). From these results and additional control experiments summarized in Fig. 3c-g, we conclude that the opposing regulatory effects of *Kr* are mediated by the sequence of the single KR *in vitro* binding motif.

To determine whether activation and repression of K-element-dependent gene expression require specific regions of KR, we examined truncated forms of the protein (Fig. 1c, d). High and low concentrations of KR lacking the 64 C-terminal amino acids could not repress, but were still able to activate gene expression (Figs 3b, 4a). Conversely, KR lacking the 116 N-terminal amino acids did not activate gene expression, but repression of gene expression was still observed (Figs 3b, 4b). These results demonstrate that distinct portions of KR other than the DNA-binding domain (Fig. 1b, c) are essential for gene activation and repression.

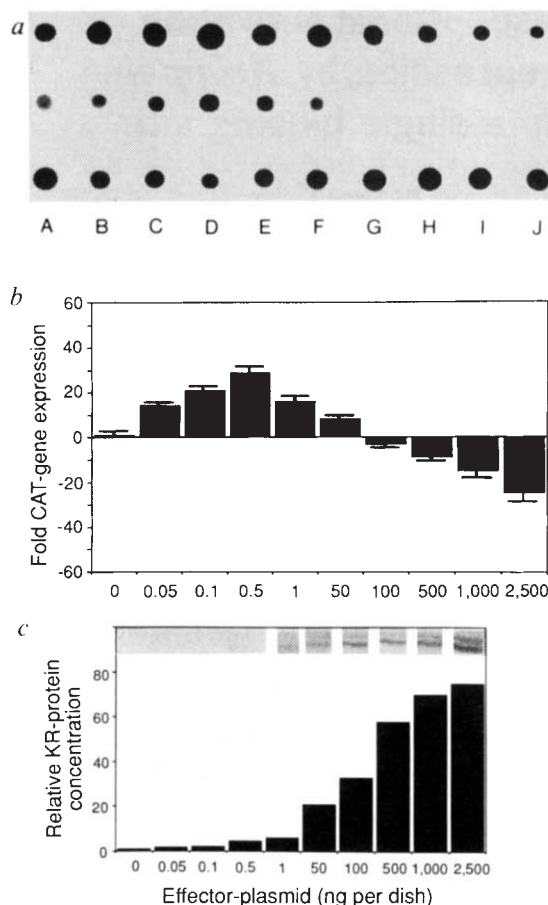


**FIG. 2** Gene expression mediated by the K-element in response to different doses of the *Kr* gene. Schneider cells were cotransfected with a constant amount of DNA (4  $\mu$ g per dish) of the reporter gene construct pP1K<sub>1</sub>KrCAT containing one K-element (see Fig. 1a), and various amounts of pPac *Kr* effector plasmid DNA (see Fig. 1b). **a**, Extracts from the transfected cells were assayed for CAT activity using <sup>14</sup>C-chloramphenicol. Autoradiographs of the reaction products show the unreacted chloramphenicol and two spots of mono-acetylated products (bottom to top). Cells were cotransfected with 0 ng (lane A), 0.05 ng (B), 0.1 ng (C), 0.5 ng (D), 1 ng (E), 50 ng (F), 100 ng (G), 500 ng (H), 1,000 ng (I), 2,500 ng (J) of pPac<sub>1</sub>Kr effector DNA. **b**, Quantitative measurements of the cotransfection experiments as shown in **a**. Note that the amount of CAT activity (shown as fold CAT gene expression relative to the basal activity of the reporter gene; black bars represent the mean value of four independent experiments; dashes indicate standard deviation) is dependent on the amount of effector DNA. KR production in response to the different amounts of cotransfected KR-expressing plasmid DNA was controlled by western blot analysis and ELISA assay using the anti-KR antibody (**c**, top and bottom).

**METHODS.** *Drosophila* Schneider S2/L3 cells<sup>28</sup> were grown to  $1-4 \times 10^6$  cells ml<sup>-1</sup> as described<sup>26</sup>. One day before the transfection, the cells were diluted to a density of  $5 \times 10^5$  cells ml<sup>-1</sup> and 5 ml aliquots of this dilution were plated onto tissue culture plates (diameter 6 cm; Nunc). After 24 h, cultures were transfected under standard conditions<sup>26</sup> with 20  $\mu$ g DNA containing a constant amount of 4  $\mu$ g reporter gene DNA, variable amounts of effector gene DNA (see abscissa) 3  $\mu$ g of pPacZ DNA (ref. 23) and variable amounts of carrier DNA (Bluescript plasmid, Strategene). Transfected cells were collected after a 60 h incubation period and processed as described<sup>26</sup>. Supernatants were assayed for  $\beta$ -galactosidase activity to account for variations of the transfections<sup>23</sup>. CAT assays and the analysis of the reaction products shown in **a** were done as described<sup>29</sup>. Quantification of CAT activity was done by the direct measurement of the CAT gene product using a commercial CAT-ELISA assay system according to the protocol provided by the manufacturer (<sup>3</sup>Prime-<sup>3</sup>Prime, West-Chester, Pennsylvania, USA). For each *Kr*-expressing gene construct (see Fig. 1) the production and nuclear location of the products were controlled using anti-Kr antibody staining as described<sup>30</sup>. The relative amount of KR produced in cotransfected cells (values shown in Fig. 2c) was controlled by western blot analysis and by the ELISA assay as outlined in ref. 31.

**FIG. 1** Fusion gene constructs used for cotransfection of *Drosophila* tissue culture cells. **a**, Reporter gene construct containing an 11-bp KR-binding site of the cis-acting control region of the *knirps* gene<sup>6,7</sup> (K-element; boxed capital letters; for details see ref. 6) flanked by linker sequences (lower-case letters), a 500-bp fragment containing the *Kr* basal promoter (open bar; transcription start indicated by arrow) and the bacterial chloramphenicol acetyl-transferase gene (hatched bar; initiation codon ATG is in position of the basal *Kr* promoter/CAT gene fusion site). **b–d**, Effector gene constructs pPac<sub>1</sub>Kr (**b**), pPacC-64Kr (**c**) and pPacN-116Kr (**d**) containing the constitutive actin 5C promoter fused to the entire (**b**) or to truncated forms of the *Kr* coding sequence (**c**, **d**) to which the 3'-untranslated region of the actin 5C gene including the polyadenylation signal had been fused. The *Kr* coding sequence is shown by an open bar, the position of the DNA-binding domain (position 221–333; ref. 2) by the filled bar. **c**, 3'-terminal sequences of the *Kr* coding region were deleted in a manner that the C-terminal 64 amino acids (position 402–466; ref. 2) are missing. **d**, 5'-terminal sequences were deleted and replaced by the sequence GATTCATGC, containing an artificial ATG initiation codon (arrow). The resulting truncated *Kr* protein lacks the N-terminal 116 amino acids (position 1–116; ref. 2). Diagnostic restriction sites are shown: *Bst*NI (B), *Hind*III (H), *Hind*III (H III), *Nde*I (N), *Pst*I (P) and *Sst*I (Ss).

**METHODS.** For the reporter gene constructs, the SV40 promoter of the pC4cat vector<sup>25</sup> was replaced by the *Kr* basal promoter (gift of M. J. Pankratz), and one or five tandem copies (*n* is 1 or 5; see **a**) of the K-element were inserted into the unique *Pst*I site in front of the *Kr* basal promoter (for orientation see **a**). For the effector gene constructs, sequences of the *Kr* pck2b complementary DNA clone<sup>2</sup> were inserted into the pPac vector<sup>26</sup> after cutting with appropriate restriction enzymes followed by filling-in reactions, and blunt-end ligations into the polished *Bam*HI site of pPac. Truncated forms of *Kr* were generated by either a *Hind*III (**c**) or a *Bst*NI digest (**d**) and inserted into the pPac vector (see above). In **d**, the blunt-ended *Bst*NI site was first fused to the linker sequence<sup>27</sup>. Fusion gene constructs containing the *Adh* promoter were identical to the construct in **a** except that the sequences of the basal *Kr* promoter were replaced by the sequences of the *Adh* promoter as described in refs 11 and 12.





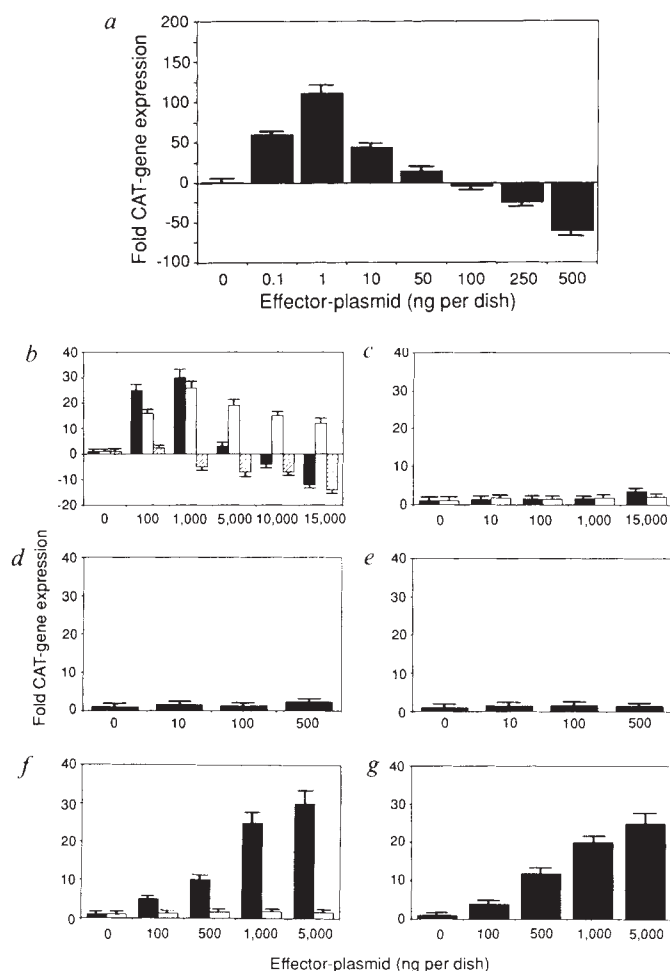
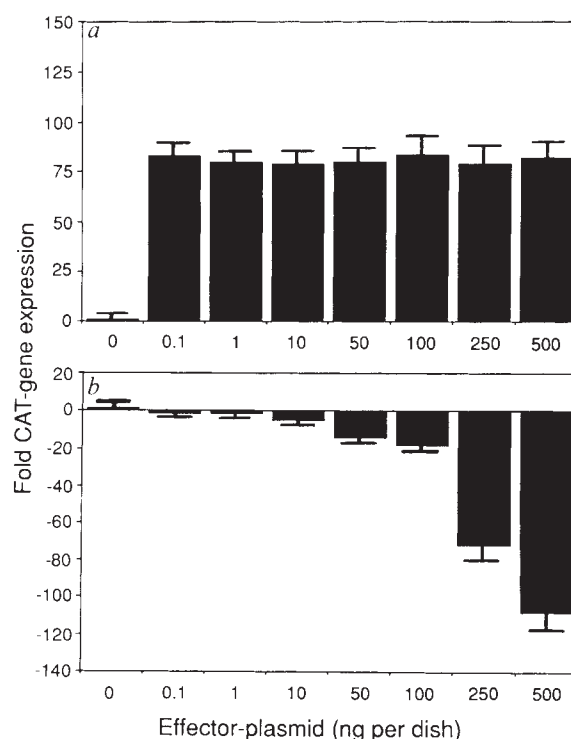


FIG. 4 Activation and repression by the *Kr* gene require different regions of the protein than the DNA-binding domain. Cotransfection experiments were performed and analysed using the CAT-ELISA assay (see legend to Fig. 2) except that truncated forms of the *Kr* gene were used as effector DNA (Fig. 1). Bars represent mean values from at least eight independent experiments, standard deviations indicated by dashes. **a**, Gene expression in response to different dosages of the pPacC-64Kr effector gene (Fig. 1c). Note activation but no repression. **b**, Gene expression in response to different dosages of the pPacN-116Kr effector gene. Note repression but no activation. Note also that the C-terminal 64 amino-acid region which provides KR repressor function contains a degenerated zinc-finger motif in which a Cys residue is replaced by a Met (position 420; ref. 2). For methods see legends of Figs 1–3.

KR can act as a transcriptional repressor both in mammalian<sup>8</sup> and in *Drosophila* tissue culture systems<sup>9</sup>. In those experiments, repression by KR was mediated by target sites which differ by at least 1 bp in the recognition sequence from the one we used<sup>8,9</sup>, and the KR binding sites were embedded in a complex array of other protein-binding sites in front of different promoter sequences<sup>9</sup>. In both experimental systems *Kr*-dependent repression may have occurred in the absence of specific KR/DNA interactions<sup>8,9</sup>. In the mammalian system, the repressor domain has been mapped to an alanine-rich N-terminal region<sup>8</sup> similar to the one observed in the transcriptional repressor *even-skipped* (refs 13, 14). In the *Drosophila* system, however, the repressor region has been mapped to the C-terminal third of KR (ref. 9) encompassing the C-terminal 64 amino-acid sequence described here. Surprisingly, the KR sequences required for gene activation as described here fully overlap the N-terminal region which carries the repressor domain described

FIG. 3 *Kr*-dependent gene expression driven by five tandem copies of the K-element. **a**, Quantitative measurements of the cotransfection experiments. Cells were cotransfected with a constant amount of the reporter plasmid DNA pP5KKrCAT (see Fig. 1a; 1  $\mu$ g per dish) and various amounts of effector DNA. CAT activity relative to the basal activity of the reporter gene (fold CAT gene expression) is indicated by black bars each representing the mean value from eight independent experiments (dashes, standard deviation). Note the *Kr*-dosage-dependent activation and repression of CAT gene expression as shown with reporter gene constructs driven by a single copy of the K-element (Fig. 2). **b**, CAT gene expression driven by five K-elements in front of the *Drosophila* Adh promoter instead of the *Kr* basal promoter. Black bars represent response to effector plasmid pPacC-64Kr (Fig. 1b), open bars represent response to effector plasmid pPacC-64Kr (Fig. 1c), and hatched bars represent response to effector plasmid pPacN-116Kr (Fig. 1d). Note that activation but no repression occurs with pPacC-64Kr, and repression but no activation occurs with pPacN-116Kr (for comparison and details see Fig. 4). **c–g**, Control experiments indicating that CAT gene expression is driven by the KR-binding site AAAAGGGTTAA in response to KR expressing DNA. **c**, No significant activation or repression of gene expression occurs with reporter gene constructs (Fig. 1a) which lack the K-element in front of either the Adh promoter (open bars) or the *Kr* basal promoter (black bar) when cells were cotransfected with various amounts of pPacC-64Kr effector DNA. **d**, No significant effect on CAT gene expression can be observed when a different transcription factor than *Kr*, in this case the gap gene *hunchback* (ref. 9), was used in the cotransfection experiments. For the results shown, pPacC-64Kr effector DNA (the *hb* coding sequence<sup>32</sup> was fused to the actin 5C promoter) was cotransfected with pPac5KKrCAT DNA under the same conditions as used in **a**. **e**, CAT gene expression from pPac5KKrCAT in response to a *Kr* expression DNA which lacks the nuclear location signal. Note no significant effect on the basal level of CAT gene expression when mutated KR is prevented from entering the nuclei. Cytoplasmic instead of nuclear location of KR was confirmed by anti-KR-antibody staining<sup>30</sup>. **f, g**, CAT gene expression mediated by a 140-bp sequence (instead of the K-element) which drives gene expression in response to the anterior morphogen *bicoid* (*bcd*) (ref. 23). **f**, Note transcriptional activation in response to increasing amounts of BCD expressing DNA<sup>23</sup> (black bars), but no effect with KR expressing DNA (open bars). **g**, No repressor function of KR when KR expressing DNA was cotransfected together with BCD expressing DNA. The results shown in **f** and **g** suggest that the sequences outside the K-element are insufficient to activate or to repress CAT gene expression, whereas the K-element is sufficient to drive gene expression in conjunction with two separate basal promoters which derive from the *Kr* and the *Adh* gene, respectively (Figs 2, 3a, b). For details see text. Bars represent mean values from at least four independent cotransfection experiments. Standard deviations are indicated by dashes. METHODS. Cotransfections and quantitative measurements of CAT gene product were done as outlined in the legend to Fig. 2 except that 6  $\mu$ g pP5KAdhCAT were used instead of 4  $\mu$ g pP1KKrCAT.



for the mammalian system<sup>8</sup>. This observation suggests the possibility that gene activation or repression by KR may involve cell-specific protein-protein interactions in different cell types. For this reason, and because the cis-acting regions of the putative *Kr* target genes contain multiple and variable KR binding sites<sup>4-6,8-10</sup>, it is not possible to foresee to which extent the regulatory potential of *Kr* as shown here is used by the embryo.

Recent studies on various transcription factors are consistent with the view that they may commonly use positive and negative regulatory activities<sup>15-20</sup>. But each activity involves a distinct set of cis-acting sequences, as in the case of the glucocorticoid receptor<sup>20</sup>. The ability of KR to effect activation and repression of transcription by its interaction with a single binding site provides a novel aspect for gene regulation in a developmental system in which the concept of concentration-dependent gene regulation seems to be of fundamental biological importance<sup>1,4,21-24</sup>. □

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## Dysmyelination in transgenic mice resulting from expression of class I histocompatibility molecules in oligodendrocytes

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**MAJOR histocompatibility complex (MHC) molecules are not normally expressed in the central nervous system (CNS)<sup>1-3</sup>. However, aberrant expression has been observed in multiple sclerosis lesions and could contribute to the destruction of myelin or the myelinating cells known as oligodendrocytes<sup>4,5</sup>. The mechanism of cell damage associated with aberrant MHC molecule expression is unclear: for example, overexpression of class I (ref. 6) and class II (refs 7, 8) MHC molecules in pancreatic  $\beta$  cells in transgenic mice leads to nonimmune destruction of the cells and insulin-dependent diabetes mellitus. We have generated transgenic mice that express class I H-2K<sup>b</sup> MHC molecules, under the control of the myelin basic protein promoter, specifically in oligodendrocytes. Homozygous transgenic mice have a shivering phenotype, develop tonic seizures and die at 15-22 days. This phenotype, which we term 'wonky', is due to hypomyelination in the CNS, and not to involvement of the immune system. The primary defect appears to be a shortage of myelinating oligodendrocytes resulting from overexpression of the class I MHC molecules.**

Using a transgenic mouse model, we have examined the hypothesis that aberrant expression of MHC molecules in oligodendrocytes may be involved in multiple sclerosis (MS). Accordingly, we linked the murine H-2K<sup>b</sup> protein-coding sequences of the genomic clone<sup>6</sup> to the 1.3-kilobase (kb) *HindIII* fragment of the murine myelin basic protein (MBP) promoter<sup>9,10</sup>. This DNA was microinjected into (CBA  $\times$  B10.BR)<sub>F2</sub> fertilized eggs and lines from six transgenic founders, backcrossed to CBA mice, were produced. Progeny heterozygous for the transgene seemed to be both clinically and histo-

logically normal. In the 81-2 and 169-6 lines, mice homozygous for the transgene, as determined by Southern analysis (Fig. 1) exhibited neurological symptoms at 11 to 14 days of age, just after the onset of myelination in normal mice. They showed intense shivering during locomotor activity, particularly in the hindquarters, and developed tonic seizures which led to death at 15-22 days. We have termed this phenotype 'wonky' (Table 1). Homozygotes in other lines did not display such symptoms. When, however, heterozygotes of the 81-2 line were crossed with heterozygotes of the 80-2 or 83-2 lines, 50% of the progeny that inherited the transgene from both parents manifested the same signs as wonky homozygotes of the 81-2 lines (Table 1). H-2K<sup>b</sup> expression, detected by immunohistochemistry, occurred predominantly in cells of the white matter and in scattered cells of the grey matter. The distribution of these cells corresponded to that of oligodendrocytes. When examined in tissue culture using

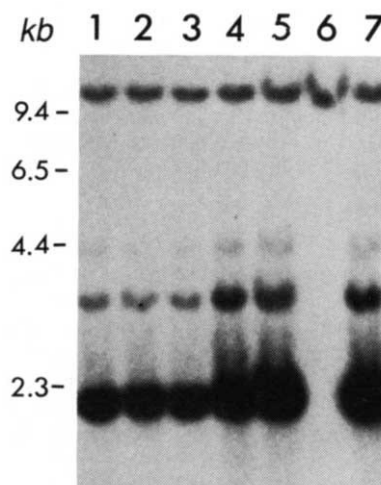


FIG. 1 Southern analysis of *Bam*HI-digested tail DNA to examine MBPp-K<sup>b</sup> transgene status of the mice. The 11-kb band corresponds to the endogenous MBP promoter, whereas the major transgene band was at 2.1 kb. Lanes 1-3 show DNA of mice heterozygous for the transgene; lanes 4, 5 and 7 show homozygous wonky mice, and lane 6, a non-transgenic. Migration of size markers (kb) is indicated on the left.

METHODS. Tail DNA was purified and Southern blotted by standard methods. The *Pst*II/*Xho*I fragment of the MBP promoter<sup>10</sup> was used as a DNA probe.



TABLE 1 Transgenic mice carrying the MBPp-K<sup>b</sup> construct

| Line        | Copy number | Total progeny of heterozygous backcrosses* | Heterozygous |              | Total progeny of brother-sister matings | Heterozygous      |              | Monozygous        |              |
|-------------|-------------|--------------------------------------------|--------------|--------------|-----------------------------------------|-------------------|--------------|-------------------|--------------|
|             |             |                                            | Number       | Number wonky |                                         | Number            | Number wonky | Number            | Number wonky |
| 80-2        | 14-16       | 85                                         | 44           | 0            | 12                                      | 6                 | 0            | 1                 | 0            |
| 81-2        | 10-12       | 130                                        | 58           | 0            | 114†                                    | 51                | 0            | 25                | 25           |
| 83-2        | 6-8         | 92                                         | 36           | 0            | 48‡                                     | 25                | 0            | 13                | 0            |
| 91-4        | 6-15        | 23                                         | 7            | 0            | ND                                      | —                 | —            | —                 | —            |
| 92-3        | 15-17§      | 129                                        | 102          | 0            | ND                                      | —                 | —            | —                 | —            |
| 169-6       | ND          | 39                                         | 13           | 0            | 38                                      | 25                | 0            | 4                 | 4            |
|             |             |                                            |              |              | Total interline cross                   | Single transgenic |              | Double transgenic |              |
|             |             |                                            |              |              |                                         | Number            | Number wonky | Number            | Number wonky |
| 81-2 × 83-2 | —           | —                                          | —            | —            | 24                                      | 13                | 0            | 8                 | 5            |
| 81-2 × 80-2 | —           | —                                          | —            | —            | 6                                       | 2                 | 0            | 3                 | 2            |

Transgene copy number was determined using a computing densitometer (Image Quant, Molecular Dynamics, USA). The wonky phenotype was characterized as intense shivering during locomotor activity, particularly in the hindquarters and the development of tonic seizures. ND, not determined.

\* Backcrossed with nontransgenic CBA or SJL.

† Six offspring exhibited the wonky phenotype but died under 3 weeks of age before DNA could be taken.

‡ Three died of unknown causes at 9, 20 and 37 days of age; 1 heterozygous, 2 with unknown genotype.

§ More than 1 integration site with rearranged transgene.

|| This line also carries the MBPp- $\beta$ 2m construct.

double indirect immunofluorescence, all transgene-expressing cells also produced the oligodendrocyte-specific marker, galactocerebroside (gal c) (Fig. 2i), although not all gal c<sup>+</sup> cells expressed H-2K<sup>b</sup>. Transgene expression was not found in peripheral nerves (sciatic) nor in spleen, kidney or thymus, but was detected in testis seminiferous tubules, intestinal villi and at low levels in the liver.

Wonky mice were killed at 12-22 days of age when they had obvious clinical symptoms. Staining of myelin lipids by luxol fast blue showed dramatically decreased myelination (Fig. 2a,

b) and MBP levels (Fig. 2c, d) compared with age-matched controls. The number of astrocytes was increased (Fig. 2e, f), indicative of a reactive gliosis. Only in the CNS was myelination affected, peripheral nerve myelination being normal (Fig. 2g, h). Tissues other than the brain and spinal cord appeared normal.

No lymphocytic infiltration was observed in any tissue in these transgenic mice. Grafting heterozygotes with skin from appropriate donors showed the mice to be tolerant of the transgene product (data not shown), as demonstrated in other

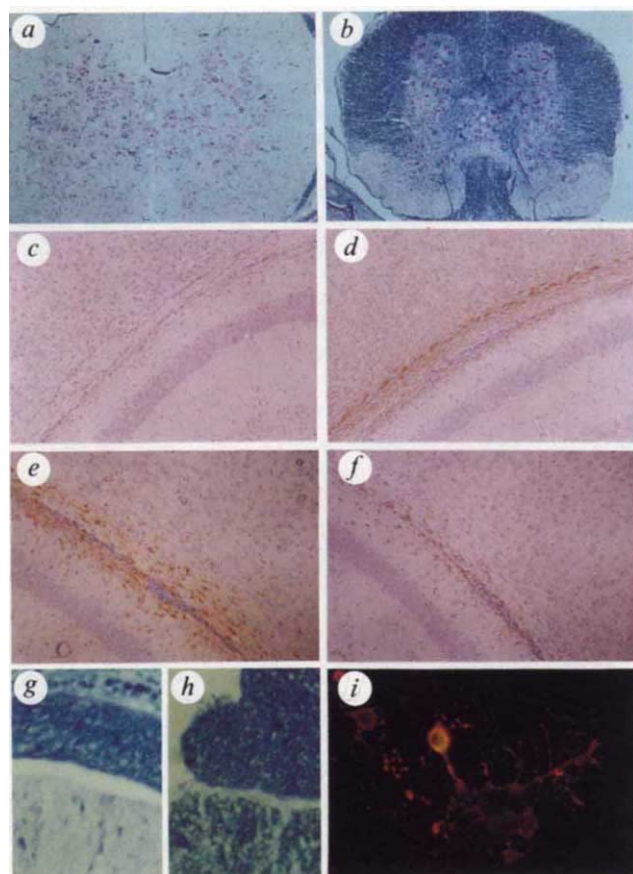


FIG. 2 Light microscope analysis of histopathological effects of transgene expression. The spinal cord of the 16-day-old wonky mouse (a) is severely hypomyelinated in comparison to a normal age-matched CBA mouse (b), as indicated by luxol fast-blue staining. Immunohistochemical examination of the corpus callosum reveals that MBP expression in the wonky mouse (c) is less than normal (d). Wonky mice had a reactive gliosis. Staining for glial fibrillary acidic protein (GFAP) is more intense and astrocyte number in the wonky mouse (e) is greater than in the normal mouse (f). Hypomyelination was not observed in the peripheral nervous system, as indicated by luxol fast-blue staining of a ganglion, adjacent to non-myelinated white matter, in the spinal cord of the wonky mouse (g). Luxol fast-blue staining of normal mouse spinal cord indicated myelination of both the ganglion and the white matter (h). In culture of transgenic mouse brain cells, transgene expression was limited to oligodendrocytes, as determined by double indirect immunofluorescence. A gal c<sup>+</sup> oligodendrocyte (red fluorescence) also expressed the H-2K<sup>b</sup> protein (green fluorescence) (i). Magnification: a, b, 50 $\times$ ; c-f, 200 $\times$ ; g, h, 500 $\times$ ; i, 800 $\times$ .

**METHODS.** Mice were perfused intracardially with normal saline followed by 10% buffered formalin. The brain and spine were removed and fixed in formalin, spines were decalcified in acid for 3-4 days and paraffin sections (1  $\mu$ m) were cut. Immunohistochemistry was as described<sup>22</sup>, MBP and GFAP being detected using a two-stage immunoperoxidase procedure with rabbit anti-human MBP<sup>23</sup> or rabbit anti-cow GFAP antisera (Dakopatts, Denmark), visualized with peroxidase-conjugated sheep anti-rabbit immunoglobulin (Silenus, Australia). Brain-cell suspensions were cultured as described<sup>24</sup> and the cells examined by indirect immunofluorescence. Oligodendrocytes were identified using monoclonal anti-gal c supernatant<sup>25</sup> and rhodamine-conjugated sheep anti-mouse immunoglobulin (Silenus, Australia). H-2K<sup>b</sup> was detected using rabbit anti-exon 8 antiserum<sup>26</sup>, which detects the H-2K<sup>b</sup> protein independently of its association with  $\beta$ 2-microglobulin, and fluorescein-conjugated sheep anti-rabbit immunoglobulin (Silenus, Australia).

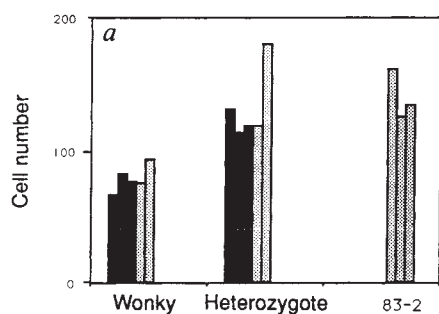


FIG. 3 Examination of oligodendrocytes using image analysis. *a*, Oligodendrocyte numbers in wonky and heterozygous mice of the 81-2 line and the 83-2 line were determined at 18 days of age. Bars with the same shading indicate mice from the same litter. *b*, Comparison of the intensity of immunofluorescence staining of the MBPp-K<sup>b</sup> transgene in oligodendrocytes from wonky mice of the 81-2 line (shaded), from heterozygotes of the 81-2 line (dashed line) and from mice of the 83-2 line (full line). For clarity, profiles of one mouse from each group are shown on the upper graph and of another mouse from each group on the lower graph. These profiles are representative of results from cells of 4 individual wonky mice and 8 unaffected mice.

**METHODS.** *a*, Mice perfused intracardially with 10% w/v sucrose had their brains removed, embedded in OCT compound (Tissue Tek) and frozen in a dry ice/hexane bath. Sections (6  $\mu$ m) were cut with a cryostat microtome, transferred to gelatin-coated slides and fixed in acetone for 10 min at 4 °C. They were stored at -20 °C and thawed for 30 s in acetone at room temperature before use. For immunofluorescence staining for H-2K<sup>b</sup> we used rabbit anti-exon 8 antiserum<sup>26</sup> diluted 1:150, visualized using fluorescein-conjugated sheep anti-rabbit immunoglobulin diluted 1:100 (Silenus, Australia). Microscopic video images of the fluorescent cells were captured by a 40 $\times$  objective lens (Zeiss) using a charge-coupled device camera, model ICD-42E (Ikegami, Japan). To capture the images the microscope stage was moved such that the tissue was sequentially examined from the top left to the bottom right side and any fields that contained oligodendrocyte/s were captured. The number of cells in each captured image was counted and the total standardized to represent the number of cells in 30

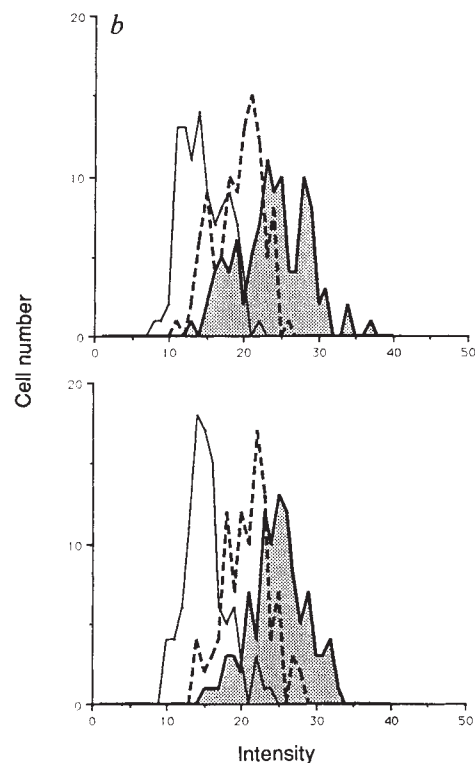


image fields. *b*, The same images as in *a* were analysed using the MD30 image analysis system (Leading Edge, Australia). The intensity parameter was used to determine the concentration of staining in each cell. Only nucleated cells were analysed to minimize differences in intensity due to the area of the cell measured. One hundred cells from each mouse were analysed.

transgenic models in which the transgenic MHC molecules are expressed in extrathymic tissues<sup>8,11-13</sup>.

To determine whether the dysmyelination in homozygous transgenic mice was associated with a decrease in oligodendrocyte number, indirect immunofluorescence was carried out on sagittal brain sections from wonky and unaffected transgenic mice and the oligodendrocytes examined using image analysis. Wonky mice had 60–70% of the oligodendrocyte number found in heterozygous mice of the 81-2 line or mice of the 83-2 line (Fig. 3*a*). The intensity of transgene staining was greater in a significant number of oligodendrocytes from wonky mice than heterozygotes of the 81-2 line, which in turn had a higher intensity than oligodendrocytes of mice from the 83-2 line (Fig. 3*b*).

We conclude that the dysmyelination observed in wonky mice was primarily due to raised intracellular levels of MHC molecules. Cell dysfunction has been associated with increased levels of transgene expression<sup>6-8,16</sup>. Oligodendrocyte death is likely to have occurred in wonky mice, since the number of these cells was less than in heterozygotes. As MBP expression is upregulated during myelination<sup>17,18</sup>, the highest levels of transgene expression probably occurred in myelinating oligodendrocytes. This population would be the most likely to undergo cell death. This could explain the profound pathological consequences, even though the reduction in oligodendrocytes in wonky mice reached only 30 to 40 per cent.

One implication of these studies for diseases such as MS is that upregulation of class I molecules alone may be sufficient to cause dysmyelination. Perhaps the high levels of MHC molecule expression necessary for damage may result from virus infection and cytokine activity. Such a nonspecific effect could explain the rapid exacerbation of disease when interferon- $\gamma$  is given to MS patients<sup>19</sup>. Thus oligodendrocytes need not be the

direct targets of either virus infections or of autoimmune responses but could be damaged as bystanders of such events. Mediation of the disease process by MHC molecules *per se* resolves the problem of failing to associate MS with a particular infectious agent or with the expression of specific autoantigens. Critical levels of MHC molecules may also be attained more easily when the heavy chain accumulates in the cytoplasm, as in our transgenic model. This may be relevant to myelinated oligodendrocytes as incorporation of newly synthesized glycoproteins into membranes of compacted myelin is very slow<sup>20</sup>, favouring raised cytoplasmic levels of molecules such as class I. This may explain why MS lesions often contain few or no oligodendrocytes among other apparently healthy non-myelinating cells<sup>21</sup>. □

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## Binding of general transcription factor TFIIB to an acidic activating region

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A CENTRAL issue in eukaryotic transcriptional regulation is the mechanism by which promoter-specific transcription factors (activators) stimulate transcription. Two lines of evidence indicate that the general transcription factor TFIIB is a pivotal component in the mechanism by which an acidic activator functions. First, during assembly of the preinitiation complex TFIIB binding is a rate-limiting step enhanced by an acidic activator<sup>1</sup>. Second, the TFIIB activity in a HeLa cell nuclear extract is specifically retained on a column containing an acidic activating region<sup>1</sup>. But because our previous study monitored only TFIIB activity, it remains possible that the interaction between TFIIB and the acidic activating region is mediated through additional proteins, for example, those designated as adaptors<sup>2</sup>, coactivators<sup>3</sup> or mediators<sup>4,5</sup>. A complementary clone encoding TFIIB has recently been isolated and shown to encode a polypeptide of relative molecular mass 35,000 (ref. 6). Here we report that TFIIB expressed in and purified from *Escherichia coli* (recombinant TFIIB) binds directly to the potent acidic activating region of the herpes simplex virus-1 VP16 protein.

TFIIB activity in a HeLa cell nuclear extract binds to a VP16 protein affinity column<sup>1</sup>. To confirm that the TFIIB activity corresponds to the cloned TFIIB polypeptide of relative molecular mass 35,000 ( $M_r$  35K)<sup>6</sup>, we assayed column fractions by immunoblotting using an antibody directed against the recombinant TFIIB polypeptide. A HeLa cell nuclear extract was applied to a VP16 protein affinity column at 200 mM KCl, the column washed extensively with 200 mM KCl, and bound proteins eluted with 1M KCl (Fig. 1). The immunoblotting results show that almost all the 35K TFIIB polypeptide is present in the 1 M KCl eluate and that only trace amounts of it can be detected in the 200 mM KCl flow-through fraction. When these same column fractions were analysed using an  $\alpha$ -TFIID antibody, the 38K TFIID polypeptide was detected solely in the 200 mM KCl flow-through. Consistent with our previous results obtained using transcription<sup>1</sup>, rather than immunoblotting, neither TFIIB nor TFIID bound to a column containing only the glutathione-S-transferase (GST) protein (data not shown, and see below). Thus, under these conditions TFIIB but not TFIID bound to an immobilized VP16 activating region.

Although the results in Fig. 1 demonstrate that TFIIB interacts with the VP16 activating region, other unidentified transcription

factors, for example TFIIB-associated polypeptides, could have been retained. We thus investigated whether recombinant TFIIB (rTFIIB) could restore transcriptional activity to a HeLa cell nuclear extract that had been depleted on a VP16 affinity column. When a HeLa cell nuclear extract was chromatographed on the VP16 affinity column, the ability of the 200 mM flow-through to support transcription directed by the acidic activator GAL4-AH was severely compromised (Fig. 2, lanes 2 and 4). Transcriptional activity was restored by addition of either the eluate of the VP16 affinity column (lane 6) or by rTFIIB (lane 8). In particular, rTFIIB restored GAL4-AH-directed transcription to an amount similar to that of the crude nuclear extract (lanes 2, 8). A longer autoradiographic exposure of Fig. 2 indicates that rTFIIB also restored the ability of the 200 mM flow-through to support the basal transcription reaction (in the absence of GAL4-AH; data not shown). Analogous results were obtained using another acidic activator, GAL4-VP16 (data not shown). Thus, under these chromatographic conditions, the 35K TFIIB polypeptide is the only general transcription factor that is absent from the flow-through fraction of a VP16 affinity column.

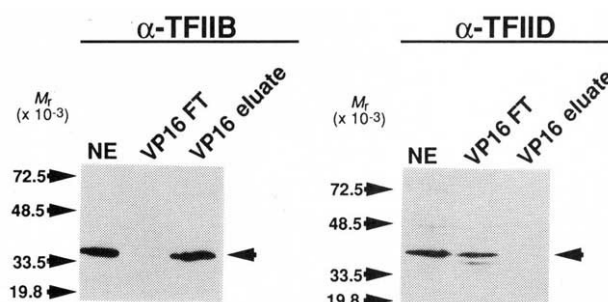


FIG. 1 The 35K TFIIB polypeptide binds to a VP16 protein affinity column. Immunoblot analysis was done using an anti-TFIIB antibody (left) or an anti-TFIID antibody (right). The samples were either 10  $\mu$ L HeLa nuclear extract (NE), 10  $\mu$ L 200 mM KCl flow-through of the GST-VP16 affinity column (VP16 FT), or 10  $\mu$ L 1 M KCl eluate of a GST-VP16 affinity column (VP16 Eluate). The positions of protein  $M_r$  standards are indicated. The positions of the TFIIB and TFIID polypeptides are indicated by arrows.

METHODS. HeLa cell nuclear extract (10 ml) was chromatographed on a 5 ml GST-VP16 affinity column as described previously<sup>1</sup>. Protein samples were fractionated on a 10% SDS-polyacrylamide gel. 1:500 and 1:1,000 dilution of the purified anti-TFIIB and anti-hTFIID antibodies, respectively, were used in the immunoblot assays. Immunodetection using enhanced chemiluminescence (ECL) western blotting detection system was done according to the manufacturer's suggestions (Amersham). To raise antibodies against rTFIIB, 200  $\mu$ g purified recombinant protein<sup>6</sup> (in 1 ml) was mixed with 1.5 ml complete Freund's adjuvant and injected into a rabbit. Three weeks later, another injection with the same amount of protein was given in complete Freund's adjuvant. Serum was obtained 2 weeks later. The anti-TFIIB antibodies were purified by incubating the serum with a 1 ml column of Affi-Gel 10 (Bio-Rad) containing 2 mg purified rTFIIB. After 90 min incubation at room temperature, resin was washed with 10 vol 20 mM Tris pH7.5, 200 mM NaCl and the antibodies were eluted with 200 mM glycine-HCl, pH2.6. Fractions containing antibodies were pooled, neutralized with 0.2 vol 1 M Tris pH7.5 and dialysed against 20 mM Tris pH7.5, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol. Anti-TFIID antibodies were prepared as follows: two synthetic oligonucleotides (5'-CCCGGGGATCCATGGATCAGAACACAGC-3' and 5'-CCCGGGGAATTCCTACGTCGTCTCCCTGAA-3') were used in a polymerase chain reaction (PCR) to amplify the cDNA encoding human TFIID (hTFIID) sequences from plasmid pKB1.04 (ref. 17). Plasmid pGLID was constructed by inserting the *Bam*HI-*Eco*RI fragment of the PCR product between the *Bam*HI and *Eco*RI sites of pGEX-2T (ref. 18). Expression and purification of the GST-hTFIID fusion protein were done as described<sup>1,18</sup>. The fusion protein was eluted with buffer A (20 mM Tris-HCl, pH7.4, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 M NaCl) containing 50 mM glutathione. Purified GST-hTFIID protein (5 mg) were injected into a rabbit. The anti-hTFIID antibodies were purified batchwise by incubating 1 ml rabbit antiserum with 1 ml GST-agarose beads<sup>5</sup> at room temperature for 20 min to remove the antibodies directed against the GST portion of the fusion protein.

Is the interaction between TFIIB and the VP16 acidic activating region direct or mediated through additional HeLa cell nuclear components? We tested whether the *Escherichia coli*-derived rTFIIB could interact with the VP16 activating region. Purified rTFIIB was chromatographed on a VP16 protein affinity column as described in Fig. 1. As with HeLa TFIIB, almost all of the rTFIIB is present in the 1 M KCl eluate of the VP16 affinity column (Fig. 3). We conclude that the 35K TFIIB polypeptide binds to the VP16 affinity column in the absence of other mammalian nuclear proteins.

The interaction between TFIIB activity and the VP16 activating region is highly specific<sup>1</sup>: a phenylalanine-to-proline substitution at position 442 of VP16 (VP16 $\Delta$ 456FP442) abolishes transcriptional activity of VP16 $\Delta$ 456 *in vivo*<sup>7</sup> and correspond-

ingly decreased binding of TFIIB *in vitro*<sup>1</sup>. We used immunoblotting to quantitate binding of HeLa TFIIB and rTFIIB to a truncated version (VP16 $\Delta$ 456) of the wild-type VP16 activating region and the phenylalanine-to-proline substitution mutant (Fig. 4). The HeLa TFIIB in a crude nuclear extract bound to the VP16 $\Delta$ 456 affinity column as evidenced by its presence in the 1 M KCl eluate. By contrast, HeLa TFIIB could not be detected in the 1 M KCl eluate of the VP16 $\Delta$ 456FP442 affinity column. This result is again consistent with our previous study<sup>1</sup> in which TFIIB activity, rather than the 35K TFIIB polypeptide, was measured. Figure 4 also shows that rTFIIB behaves similarly to HeLa TFIIB: binding of rTFIIB was much greater to the wild type than to the mutant VP16 affinity column.

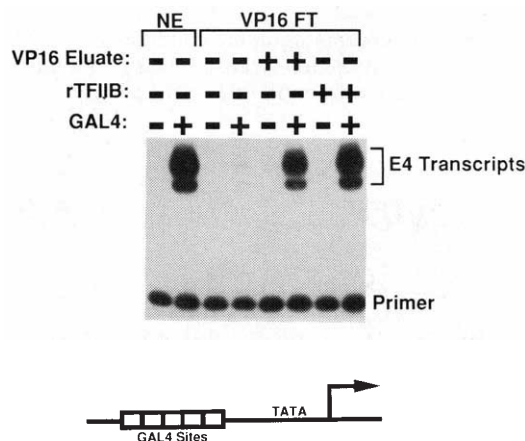


FIG. 2 The rTFIIB restores transcriptional activity to the 200 mM KCl flow-through of a VP16 affinity column. Transcription was done either in a crude HeLa cell nuclear extract (NE) or the 200 mM KCl flow-through of a VP16 affinity column (VP16 FT). The plasmid pG<sub>5</sub>E4T (ref. 19) was used as a template. The presence (+) or absence (–) of GAL4-AH, the 1 M KCl eluate (10  $\mu$ l) of the VP16 column eluate, or purified rTFIIB (13 ng) is indicated above each lane. Transcripts were quantitated by primer extension. A schematic diagram of the DNA template is shown below the autoradiogram. METHODS. Purification of GAL4-AH, fractionation of a HeLa cell nuclear extract over a GST-VP16 protein affinity column, and all other methods were as described<sup>1,19</sup>. The rTFIIB was purified by phosphocellulose and DEAE-cellulose chromatography as described<sup>6</sup>.

FIG. 3 Direct binding of rTFIIB to the VP16 protein affinity column. As in Fig. 1 except that rTFIIB was used. Lane 1, purified rTFIIB (5  $\mu$ l); lanes 2 and 3, 200 mM KCl flow-through (10  $\mu$ l) and 1 M KCl eluate (10  $\mu$ l) of the GST-VP16 protein affinity column, respectively.

METHODS. rTFIIB (500  $\mu$ l at 130  $\mu$ g ml<sup>-1</sup>) in buffer D (ref. 20) containing 200 mM KCl was loaded on a 1 ml GST-VP16 protein affinity column. After collecting the flow-through (about 1.2 ml), the column was washed extensively with 4 ml buffer D containing 200 mM KCl, and eluted with buffer D containing 1 M KCl. All other methods were as described in Fig. 1.

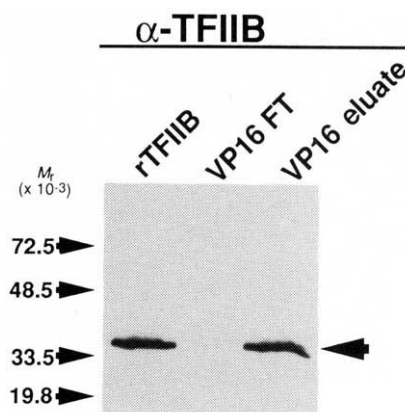
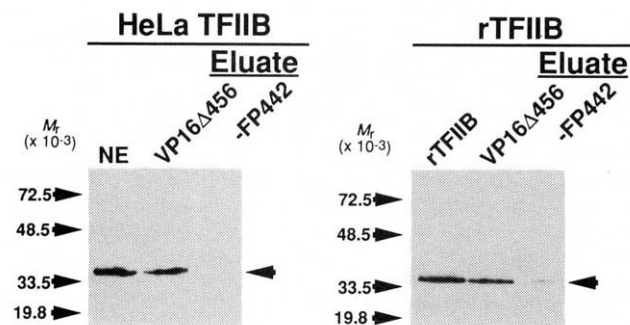


FIG. 4 Preferential binding of rTFIIB to a wild-type versus a mutant VP16 protein affinity column. As in Fig. 1. Immunoblot analysis of TFIIB from the 1 M KCl eluate of GST-VP $\Delta$ 456 and GST-VP $\Delta$ 456FP442 protein affinity columns. HeLa cell nuclear extract (left) or an *E. coli* extract containing rTFIIB (right). Lane 1, crude extracts (10  $\mu$ l); lanes 2 and 3, the eluate (10  $\mu$ l) from the GST-VP $\Delta$ 456 and GST-VP $\Delta$ 456FP442 protein affinity columns, respectively.

METHODS. Preparation of and chromatography of a HeLa cell nuclear extract over GST-VP $\Delta$ 456 and GST-VP $\Delta$ 456FP442 protein affinity columns were as described<sup>1</sup> except that protein samples were applied at 120 mM KCl. An *E. coli* extract from *E. coli* strain BL21 was transformed with pHLIB (ref. 6) and induced according to published procedures<sup>6</sup>. Bacteria were sonicated in 1/20th culture volume of buffer A. Insoluble debris was removed by centrifugation (10,000 g, 10 min); the supernatant was dialysed against buffer D containing 120 mM KCl. Bacterial extract (10  $\mu$ l; 2.8 mg protein ml<sup>-1</sup>) were diluted into 1 ml buffer D containing 120 mM KCl and 2 mg ml<sup>-1</sup> BSA and loaded on 1 ml of either a GST-VP $\Delta$ 456 or GST-VP $\Delta$ 456FP442 protein affinity column. The columns were washed with 4 ml buffer D containing 120 mM KCl and bound proteins were eluted with buffer D containing 1 M KCl. All other methods were as described in Fig. 1.





We have demonstrated here a direct and specific protein-protein interaction between the 35K TFIIB polypeptide and the VP16 acidic activating region. These results, together with those from our previous study<sup>1</sup>, argue strongly that TFIIB is a direct target of an acidic activating region. Several studies have suggested that there is a set of transcription components, referred to as adaptors<sup>2</sup>, coactivators<sup>3</sup> or mediators<sup>4,5</sup> that are required for transcription directed by an activator but not for the basal transcription reaction. These components may be the direct targets of activating regions<sup>2-5</sup> (reviewed in ref. 8). Our finding of a direct interaction between the 35K TFIIB polypeptide and the VP16 activating region argues that acidic activators do not use a coactivator to perform such a 'bridging' function. But our results do not exclude the possibility that there may be components in addition to the known general transcription factors required to support the maximal level of transcription directed by an acidic activator (for example TFIID-associated polypeptides). Furthermore, transcription factors with non-acidic activating regions may work through intermediary proteins. The HSV-1 VP16 protein itself is probably an example of such an intermediary protein, which works in conjunction with the cellular transcription factor Oct-1 (ref. 9, reviewed in ref. 10).

It will be important to determine the region of TFIIB that mediates the interaction with the VP16 activating region. TFIIB

amino acids 185-202 have the potential to form an amphipathic  $\alpha$  helix with five uninterrupted basic residues on one side<sup>6</sup>. TFIID, which appears to interact with an immobilized VP16 activating region<sup>1,11,12</sup>, also has a predicted amphipathic  $\alpha$ -helix (residues 133-145)<sup>13</sup>.

An important property of eukaryotic transcriptional activators is that they can cooperate with one another to stimulate transcription synergistically (reviewed in ref. 14). Even a very strong activator, such as GAL4-VP16, requires at least two binding sites on a target promoter to stimulate transcription efficiently<sup>15</sup>. For a weak activator, as many as five binding sites are required to achieve a high level of transcription<sup>15</sup>. This cooperativity can be shown *in vitro* to be independent of DNA binding<sup>15,16</sup>. A proposed model that accommodates these observations is that synergism results from the simultaneous contact of a common target by multiple activators<sup>15,16</sup>. But neither the amino-acid sequence of TFIIB (ref. 6) nor that of TFIID (ref. 13) reveals any obvious repeat that would accommodate up to five activating regions. Alternatively, during preinitiation complex assembly more than one transcription component may be contacted by the bound activators<sup>1</sup>. This latter model is consistent with the findings that factors in addition to TFIIB have been reported<sup>1,11,12</sup>, or proposed<sup>2-5</sup> (reviewed in ref. 8), to interact with activating regions. □

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## A new human HLA class II-related locus, *DM*

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**HLA CLASS II molecules have a crucial role in the immune response to antigens. We have isolated two new class II-like complementary DNA sequences, *RING6* and *RING7*, which map between the *HLA-DNA* and *-DOB* loci. They are novel members of the immunoglobulin gene family which may have diverged before the duplications that gave rise to the main class II loci. The *RING6* and *RING7* genes seem to encode  $\alpha$ - and  $\beta$ -chains of a previously undiscovered class II-related protein.**

New class II-related sequences were identified by screening mouse MHC cosmid<sup>1</sup>. Using probes 5.22D and 5.22C, corresponding human cDNA clones, called *RING6* and *RING7*, respectively, were obtained. These genes mapped close to each other in the MHC class II region (Fig. 1a), at least 50 kilobases (kb) and 80 kb from *DNA* and *DOB*, respectively, and separated from them by other non-class II genes, *RING3* and *RING4* (ref. 2). Given that  $\alpha$  and  $\beta$  chains of all other class II gene loci are closely associated (for example *DQA* and *DQB*), the new genes are presumably not partners for *DNA* and *DOB*.

*RING6* and *-7* both detected 1.2-kb transcripts in total B cell RNA but expression was not detected in T-cell lines (Fig. 1b, c). Treatment of epithelial and fibroblast cell lines with inter-

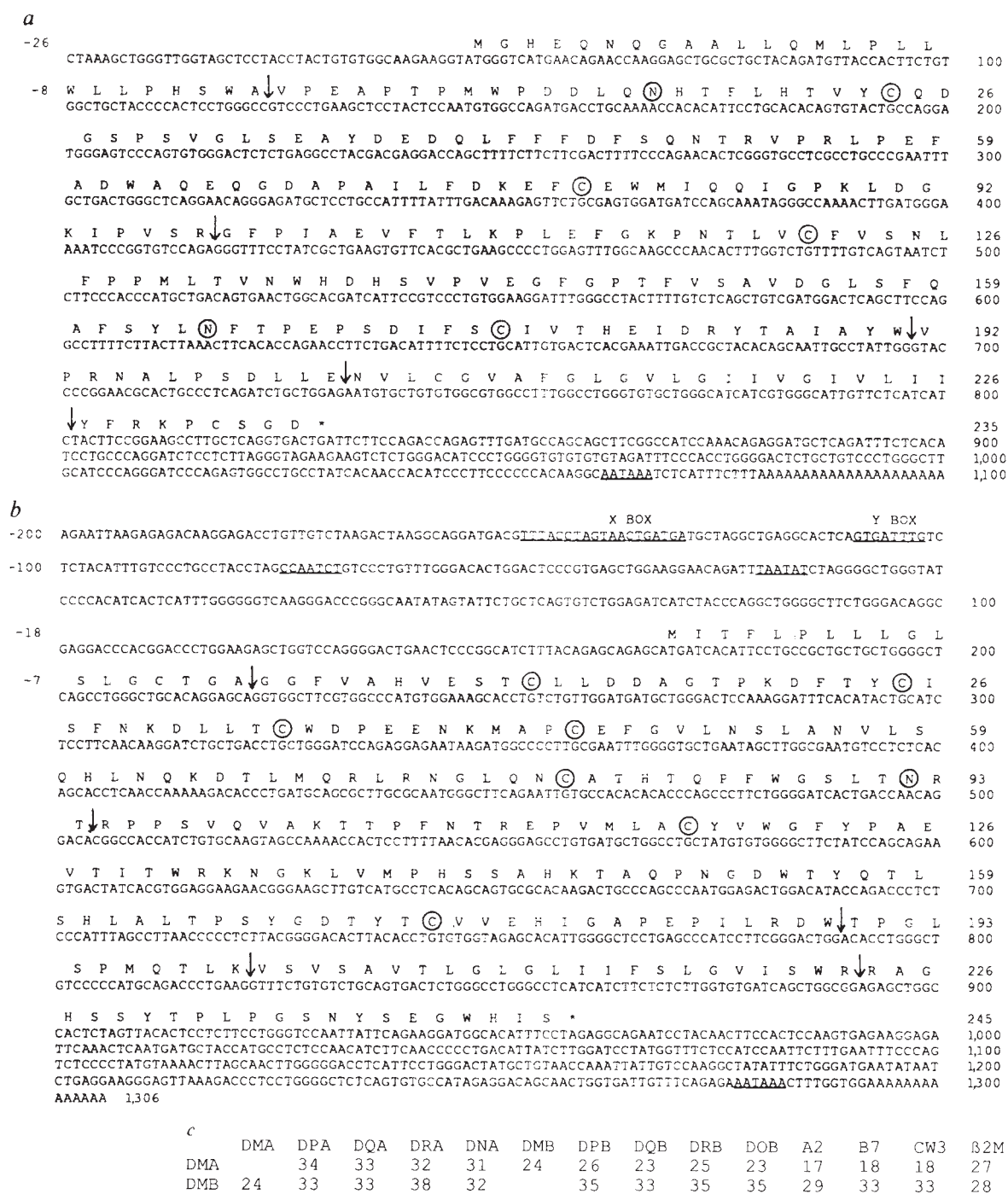
feron- $\gamma$ , which induced *DR* messenger RNA, also resulted in *de novo* *RING6* and *-7* expression.

*RING6* potentially encodes a protein of 261, and *RING7* 263, amino-acids (Fig. 2a, b), of domain configurations similar to those of a class II heterodimer. The  $\alpha 2$  domain of *RING6* was more related to class II  $\alpha$  chains and  $\beta_2$ -microglobulin ( $\beta 2M$ ) than to class II  $\beta$  chains or class I, consistent with MHC evolutionary trees which place class II  $\alpha$  and  $\beta 2M$  on the same branch (Fig. 2c; see refs. 1, 3). *RING7* was equidistant from several different immunoglobulin superfamily sequences including class I and II (Fig. 2c), but it contained short motifs characteristic of class II  $\beta$ -chain sequences<sup>1</sup>. In addition, *RING6* and *RING7* genes both had typical class II ( $\alpha$  or  $\beta$ ) promoter elements but the spacing between the X and Y elements was like class II  $\alpha$  (19 base pairs (bp)) in both cases (Fig. 2d)<sup>4</sup>. This may be the primordial arrangement, a 1-bp deletion having occurred in class II  $\beta$  before duplication of the main class II loci. The *RING6* and *-7* genes have sufficient similarity with conventional class II  $\alpha$  and  $\beta$  sequences, respectively, that we suggest that they be termed *HLA-DMA* and *-DMB*.

The proposed *RING6/7* protein heterodimer is very different to other class II molecules in the membrane distal domains. *RING6* contains two cysteine residues in this domain and there are five cysteines in the equivalent region of *RING7*. Initial modelling data predicts an antigen-binding cleft with a conformation similar to that proposed for *DR* (ref. 5), but with the cysteine residues forming disulphide bridges, making the structure more rigid, with restricted peptide binding capacity (not shown).

The *RING6/7* structure may act like a conventional class II molecule or may have a novel role, dedicated to presenting a particular moiety. A precedent for this is the divergent class I

FIG. 2. Nucleotide and derived amino-acid sequences (single-letter code) of *RING6* (a) and *RING7* (b) cDNA inserts. Numbering of amino acids starts from the putative signal sequence cleavage points and proposed boundaries between signal sequence, membrane proximal/distal, connecting peptide, transmembrane and cytoplasmic domains are indicated by arrows. Stop codons are marked by \* and polyadenylation signals are underlined. Potential N-linked glycosylation points and cysteine residues described in the text are circled. Note that the glycosylation sites are in different positions to those found in other class II  $\alpha$ - and  $\beta$ -chains. Nucleic acid sequence is numbered from the start of the cDNA (confirmed as the transcription initiation points by primer extension; data not shown). The additional sequence (negative numbering) 5' of the *RING7* cDNA is from genomic DNA and extends into the promoter region. Putative X and Y box elements as well as CAAAT and TATA box homologies are underlined. Without glycosylation, assuming the signal



sequence is cleaved where suggested, the mature RING6 protein would have a predicted relative molecular mass of 26,200 and a pl of 4.1 and the RING7 protein a molecular mass of 27,100 and a pl of 7.2. *c*, Amino-acid (%) identity in the membrane proximal domain of RING6 (DMA) and RING7(DMB) with other MHC class II  $\alpha$  and  $\beta$  sequences, three representative class I sequences and  $\beta$ 2M. Sequences were taken from published papers as follows DPA, DQA, DRA, DNA (ref. 12), DPB, DQB, DRB, DOB (ref. 13) A2, B7 and CW3 (ref. 14)  $\beta$ 2M (ref. 15). Note that DMA and DMB are not closely related to each other (24%) and that DMB is as related to class I  $\alpha$ 3 and class II  $\alpha$ 2 sequences as it is to class II  $\beta$ 2. *d* Comparison of X and Y box elements from class II human and mouse genes. Identity with the consensus elements is indicated by a dash, Y=T/C and R=G/A. The number of nucleotides separating the elements is as indicated for each sequence. Promoter sequences are from published papers as follows DPA, DRA, DNA, Ea, DPB, DQB, Ab and Eb (ref. 4); DQA1, DQA2, Aa, DPB2, DQB2, DRB and DOB (ref. 16). Nucleic-acid sequencing was done by random dideoxynucleotide chain termination<sup>17</sup> and the SAP program was used for sequence assembly<sup>18</sup>

| <i>d</i>  | X box                                      | Y box        |
|-----------|--------------------------------------------|--------------|
| Consensus | TYTNCCYAGNRACAGATGA <--18/19 BASE PAIRS--> | CTGATTGGYY   |
| RING6     | -----T-----                                | -----        |
| RING7     | -----T-----                                | G-----T----- |
| DPA       | -----GA-----                               | -----A-----G |
| DQA       | --C-G-----T--GAT                           | -----A-----  |
| DQA 2     | -GC-A-G---ACA--AT                          | -----        |
| DRA       | --C-----C-----                             | -----        |
| DNA       | -----AC-----                               | G-----       |
| Ea        | C-----T-----                               | -----        |
| Aa        | -GCAG--G---G-TGAC                          | -----        |
| DPB       | -----GCA-----                              | TCC-----     |
| DPB 2     | -----GCA-----                              | -CC-----     |
| DQB       | -----T-----                                | -----        |
| DQB 2     | -----G-----                                | -----        |
| DRB       | -----A-----T-----                          | -----        |
| Eb        | -----A-----T-----                          | -----        |
| Ab        | -----C-----                                | -----        |
| DOB       | C-----C-T-----                             | T-----       |



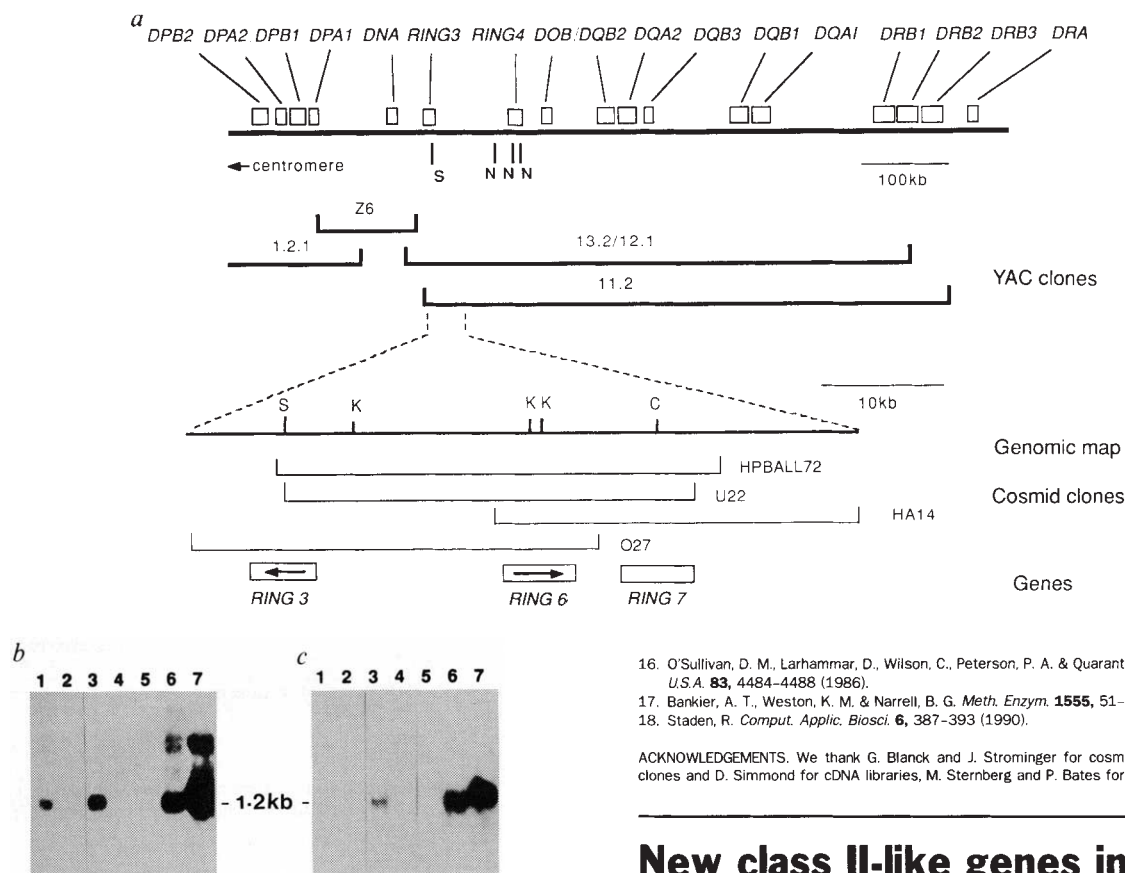


FIG. 1 a, Location of the *RING6* and *RING7* genes in the class II region of the MHC on the short arm of chromosome 6. The upper diagram shows the main genes. Landmark *SalI* and *NotI* sites are specified by S and N, respectively. *RING3* and *RING4* genes have been described previously<sup>2,8</sup>. Below the map, YAC clones spanning the region are presented as horizontal bars. The large-scale map below indicates the region encompassing the *RING6* and -7 genes positioned in respect to cosmid clones<sup>9,10</sup>. Northern blots probed with *RING6* (b) and -7(c) cDNA inserts. Lanes 1 and 2, SV80 (fibroblast) + and -IFN $\gamma$ , respectively; lanes 3 and 4, SW1222 (colon carcinoma) + and -IFN $\gamma$ , respectively; lane 5, Molt 4 (T cell); lane 6, Raji (B cell); and lane 7, DX3 (melanoma). Interferon treatment was with 250 units IFN $\gamma$  for 24 h. Total mRNA was prepared by guanidium/caesium chloride ultracentrifugation and separated by formaldehyde gel electrophoresis<sup>11</sup>. Hybridization and northern membrane transfer procedures were as specified by Amersham (Membrane Transfer and Detection Methods).

mouse locus which presents an *N*-formyl-methionine mitochondrial peptide<sup>6,7</sup>. As the *RING6* and -7 genes are remarkably different to other class II sequences and yet highly conserved between human and mouse<sup>1</sup>, it seems likely that they perform an important function which possibly predates divergence of class I and class II.

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## New class II-like genes in the murine MHC

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**MAJOR histocompatibility complex (MHC) class I molecules present endogenous antigens to CD8<sup>+</sup> (cytotoxic) T cells. MHC class II molecules present primarily exogenously derived antigens to CD4<sup>+</sup> T cells. Three new genes (*Ma*, *Mb1* and *Mb2*) located between the *Pb* and *Ob* genes of the murine MHC have properties indicating that they are members of the MHC class II gene family, but they are the most divergent class II members so far identified and are almost as closely related in sequence to class I genes as they are to the known class II genes.**

The *Ma* gene is ~60 kilobases (kb) telomeric of the *Pb* gene, and two copies of the *Mb* gene (*Mb1* and *Mb2*) are found ~5 kb telomeric to *Ma* (ref. 1). The sequences of both *Ma* and *Mb* complementary DNA clones (Fig. 1) contain single long open reading frames of 261 amino acids. The predicted product of the *Ma* gene (after removal of the putative leader peptide<sup>2</sup>) has a relative molecular mass ( $M_r$ ) of ~25,900 (25.9K) and a pI of 4.2. The *Mb* sequence predicts a protein of  $M_r$  27.0K and pI of 7.1.

The predicted *Ma* and *Mb* proteins are similar to the known class II  $\alpha$  and  $\beta$  chains, respectively, both in primary amino-acid sequence and in that they are predicted to be integral membrane proteins with two external domains, where the membrane proximal domain is homologous to immunoglobulin (Fig. 2). Both the *Ma*  $\alpha 2$  and *Mb*  $\beta 2$ -like domains have the conserved pair of cysteine residues and invariant tryptophan present in all immunoglobulin-superfamily members. *Mb* contains the highly conserved residues diagnostic of class II  $\beta$  chains at position 167-171 (NGDWT in one-letter amino-acid code) and 188-193 (GD/EVYTC). Their transcriptional polarity, tissue distribution, and regulation by interferon- $\gamma$  (refs 1, 3) also seem to be

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identical with other class II  $\alpha$ - and  $\beta$ -chain genes. As for all  $\beta$ -chain genes<sup>4</sup>, messenger RNA splicing occurs between the first and second nucleotides of a codon, and each external domain of *Mb* is encoded in a separate exon (Fig. 1b).

Several properties distinguish these new genes from the classical class II genes, however. First, the membrane distal domains of *Ma* and *Mb* are only marginally related (14–25%

amino-acid identity) to other class II genes. Interestingly, the *Mb*  $\beta$ 1 domain includes five cysteine residues, all of which are conserved in identical positions in the human homologue, RING 7 (ref. 3). Second, the membrane-proximal immunoglobulin-like domains are not as related to the known class II chains (31–39%) as the latter are to one another (54–85%). Third, the potential glycosylation sites in *Ma* and *Mb* do not coincide

FIG. 1 DNA and predicted amino-acid sequence of *Ma* (a) and *Mb* (b) cDNA clones. The putative leader peptide is double-underlined. Cysteine residues are shaded. Potential N-linked glycosylation sites are boxed. Termination codons are marked as asterisks (the upstream in-frame termination codons indicate that the protein sequence shown is complete). The polyadenylation signal sequence is underlined. The predicted boundaries between domains are shown as filled triangles. 5UT, 5'-untranslated region; LP, leader peptide; CP, connecting peptide; TM, transmembrane portion; CY, cytoplasmic domain; 3UT, 3' untranslated region. The *Ma* cDNA clone, 5.22DII-1, was isolated from a WEHI-3 cDNA library using the genomic probe, 5.22DII, a 2.6-kb *Bam*HI subclone isolated from the 5.22D *Eco*RI fragment<sup>1</sup>. The *Mb* cDNA clone, 5.22CIII-2, was isolated from the WEHI-3 library using the 5.22CIII genomic probe, a 3.1-kb *Bam*HI fragment derived from the 5.22C *Eco*RI fragment<sup>1</sup>. Exon-intron boundaries were determined by sequencing through them on one strand using cloned genomic BALB/c DNA as template and synthetic oligonucleotide primers derived from the cDNA sequence.

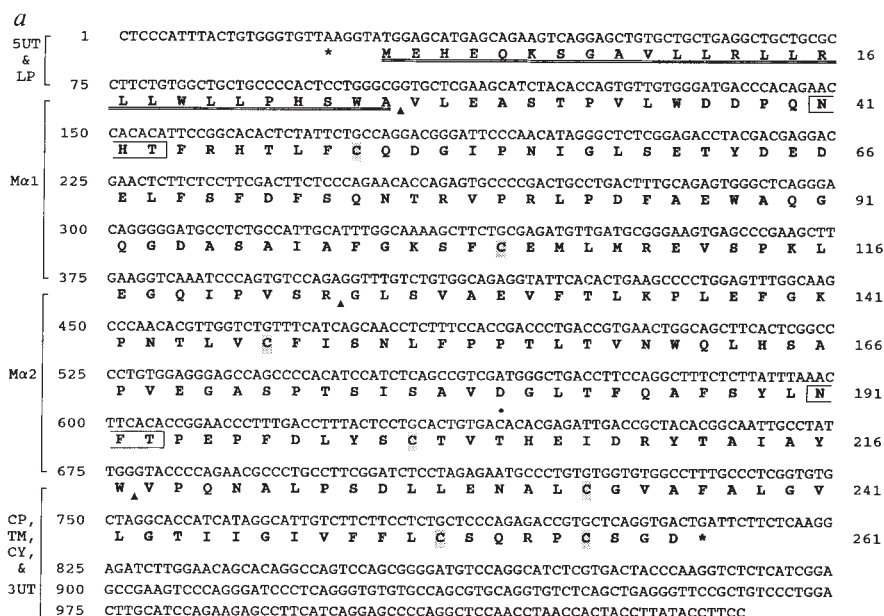
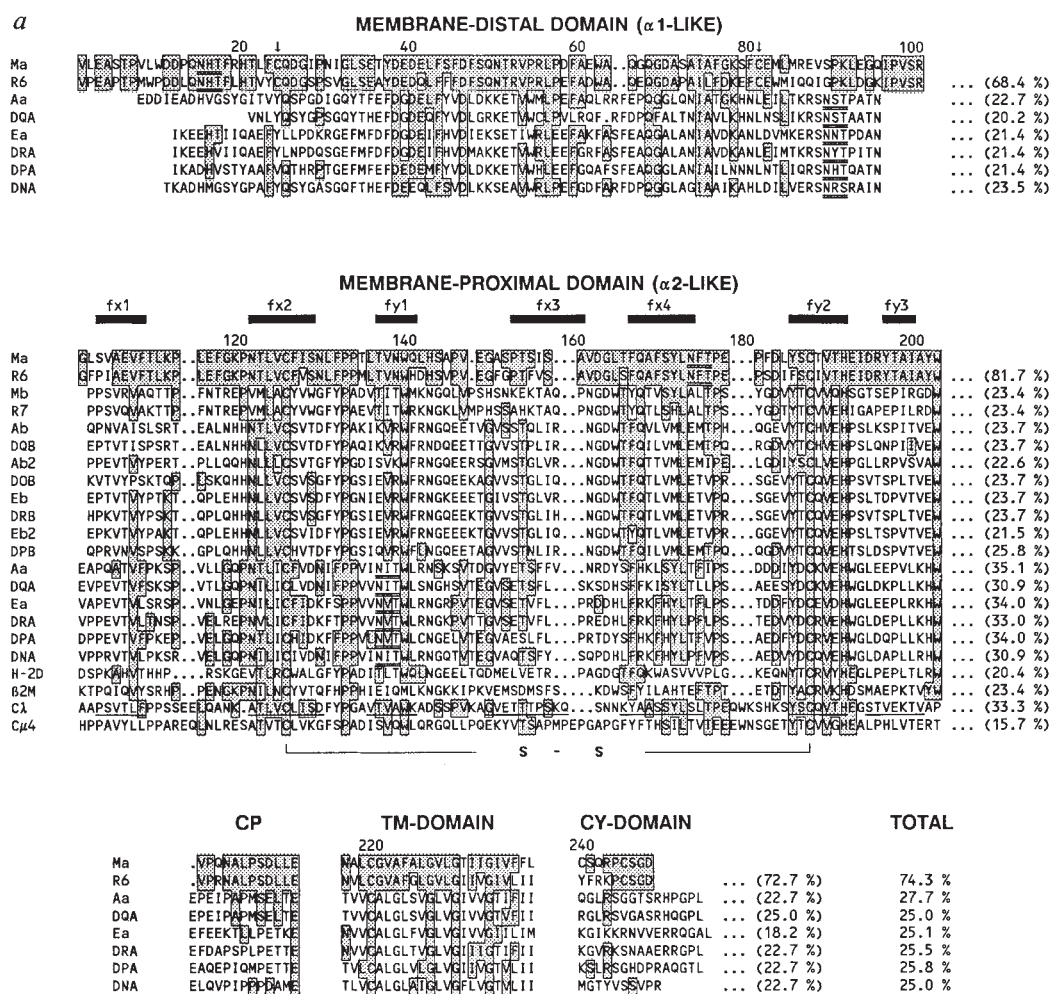


FIG. 2 Amino-acid sequence (single-letter code) comparisons of the *Ma* (a) and *Mb* (b) proteins with other MHC antigens and immunoglobulin molecules. Gaps introduced to maximize alignments are marked as dots; alignment of  $\alpha$ 1 and  $\beta$ 1 domains is tentative, owing to the low similarity of these domains to other family members. Amino acids identical to those found at the same position in *Ma* (a) and *Mb* (b) are indicated by shaded boxes. Disulphide bridges are marked as a line connecting two cysteine residues. Potential N-linked glycosylation sites are double-underlined. The regions of  $\beta$  strands determined by X-ray crystallography of C $\lambda$  are underlined, and predicted  $\beta$ -strand regions of *Ma* and *Mb* proteins are marked as black bars at the top of the sequences and designated as fx1–4 and fy1–3. The per cent amino-acid identity of *Ma* and *Mb* domains to the corresponding domains of immunoglobulin superfamily members is shown at the end of each line. Total identity is given in the last column, next to CP–TM–CY identity. The amino-acid sequences are from refs 6–23 respectively: Aa, Ea, DPA, DQA, DRA, Ab, Ab2, Eb2, DPB, DQB, DRB, DOB, H-2D, murine  $\beta$ 2-microglobulin, murine IgC $\mu$ 4, human IgC $\alpha$  (New).





|     | CP         | TM-DOMAIN             | CY-DOMAIN               | TOTAL        |
|-----|------------|-----------------------|-------------------------|--------------|
|     |            | 220                   | 240                     |              |
|     |            |                       | 260                     |              |
| Mb  | TPGLSPDITM | VSVSAAITLGLGIIHVGFRH  | PKSHSESYTFLSGSTHGRH     |              |
| R7  | TPGLSPDITL | VSVSAAITLGLGIIHSLVTSV | PRAGHSYTFPLSGNSEGHNIS   | ... (67.9 %) |
| Ab  | RAQESARS   | MLSGIGGVFLGLGLGIIH    | RSQKGRGPFAGLLQ          | ... (16.3 %) |
| DQB | RAQESARS   | MLSGIGGVFLGLGLGIIH    | RSQKGLLH                | ... (17.5 %) |
| Ab2 | MAQESYWK   | ILSGAAVFLGLGLGVVHIL   | KAQKASVETDPGNE          | ... (21.3 %) |
| DOB | RAQESYWK   | MLSGIAFLGLGLGLGIIH    | RAQKGYRTGSGNEVSRVLLPQSC | ... (21.2 %) |
| Eb  | KAQTSQNK   | MLSGVGGFVLGLFLGAGLIYF | RNKGQSGCLPTGLLS         | ... (18.8 %) |
| DRB | RARSQAQK   | MLSGVGGFVLGLFLGAGLIYF | RNKGHSGCLPTGRFLS        | ... (18.8 %) |
| Eb2 | RARSTSAQNK | LLSGVMGMLGLFLAGLIYF   | RNLH                    | ... (25.0 %) |
| DPB | KAQDSARS   | TLTGAGFVLGLGLGVIMHR   | RSKKVQRGSA              | ... (25.6 %) |

mice devoid of functional I-A or I-E molecules still have some peripheral CD4<sup>+</sup> T cells<sup>5</sup>. Such cells may be selected by the products of the genes described here. □

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## Mobile reactive centre of serpins and the control of thrombosis

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Two protease inhibitors in human plasma play a key part in the control of thrombosis: antithrombin inhibits coagulation and the plasminogen activator inhibitor PAI-1 inhibits fibrinolysis, the dissolving of clots. Both inhibitors are members of the serpin family and both exist in the plasma in latent or inactive forms. We show here that the reactive centre of the serpins can adopt varying conformations and that mobility of the reactive centre is necessary for the function of antithrombin and its binding and activation by heparin; the identification of a new locked conformation explains the latent inactive state of PAI-1. This ability to vary conformation not only allows the modulation of inhibitory activity but also protects the circulating inhibitor against proteolytic attack. Together these findings explain the retention by

the serpins of a large and unconstrained reactive centre as compared to the small fixed peptide loop of other families of serine protease inhibitors.

The serine protease inhibitors all function by forming tight complexes with their target proteases. This is achieved by having a reactive centre that acts as an ideal substrate with a P<sub>1</sub>–P<sub>1'</sub> site, matching the protease's specificity, held in an exposed position on a peptide loop. An apparent requirement is that this loop should have a defined 'canonical' structure as superimposable loop conformations have independently evolved in each of the diverse families of serine protease inhibitors<sup>1</sup>. The exception is the serpins<sup>2</sup>, which have a much larger reactive centre loop extending on the amino-terminal side of the reactive centre from P<sub>1</sub>–P<sub>14</sub>, compared with P<sub>1</sub>–P<sub>5</sub> in the canonical structure. The extra sequence enables the reactive-centre loop of the serpins to adopt varying conformations; to simplify discussion the known and deduced structures are depicted in Fig. 1 with the loop (in red) shown as it enters and leaves the dominant feature of the molecule, the five-stranded A  $\beta$ -pleated sheet (in blue).

The external position of the reactive centre loop makes it vulnerable to proteolytic cleavage and the serpins are readily inactivated in this way by a range of bacterial, snake venom

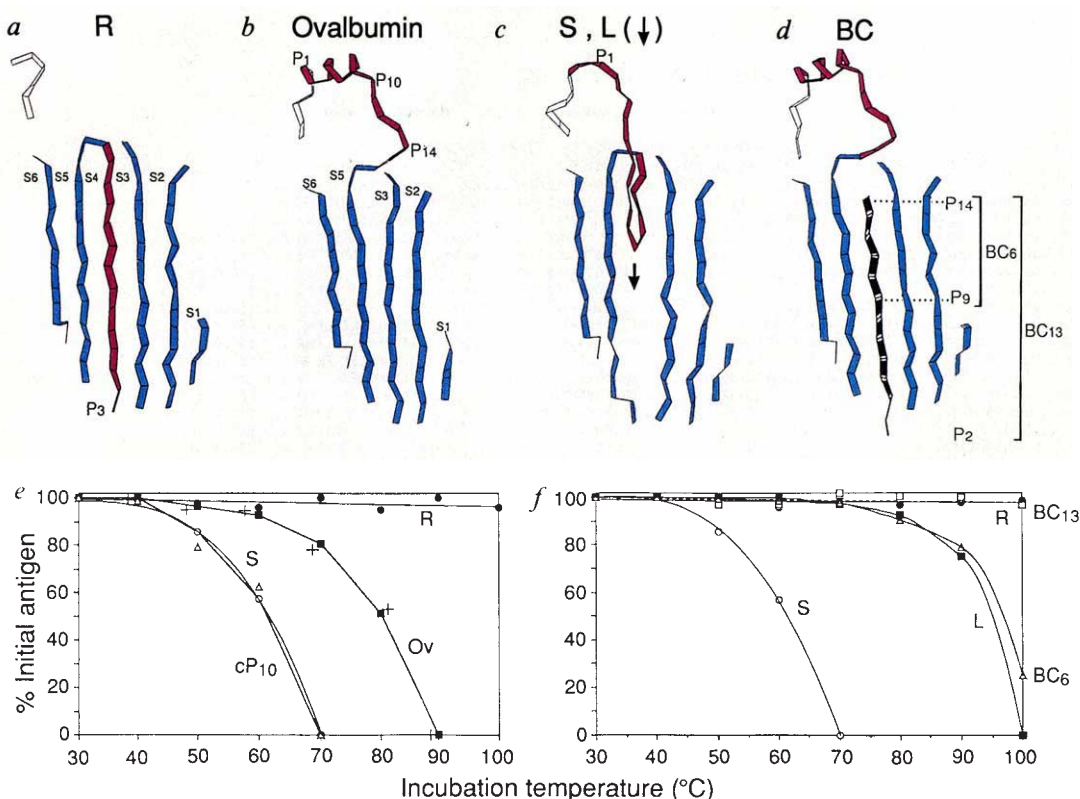




TABLE 1 Reactive centre loop sequences<sup>1</sup>

|                              | P14 | 13 | 12 | 11 | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 |
|------------------------------|-----|----|----|----|----|---|---|---|---|---|---|---|---|---|
| Serpin consensus             | T   | E  | A  | A  | A  | T | X | X | X | X | X | X | X | - |
| Antithrombin                 | S   | E  | A  | A  | A  | S | T | A | V | V | I | A | G | R |
| Antithrombin P <sub>10</sub> | S   | E  | A  | A  | P  | S | T | A | V | V | I | A | G | R |
| Ovalbumin                    | R   | E  | V  | V  | G  | S | A | E | A | G | V | D | A | A |
| Angiotensinogen              | R   | E  | P  | T  | E  | S | T | Q | Q | L | N | K | P | E |
| BC <sub>13</sub> (synthetic) | AcS | E  | A  | A  | A  | S | T | A | V | V | I | A | G |   |
| BC <sub>6</sub> (synthetic)  | AcS | E  | A  | A  | A  | S |   |   |   |   |   |   |   |   |

Sequences are shown in one-letter code. The boxed region is critical for refolding (Fig. 1) and is conserved in all inhibitory serpins but not the non-inhibitors ovalbumin, angiotensinogen, and non-functional mutants such as P<sub>10</sub> antithrombin.

and other proteases<sup>3</sup>. Cleavage of the reactive centre results in a profound structural change<sup>4</sup>, with the peptide loop being inserted into the middle of the A-sheet to give a more stable relaxed R form of the molecule (Fig. 1a). This change on cleavage from the native stressed S form to the thermostable R form (S→R) (Fig. 1e), is a characteristic feature of the inhibitory serpins<sup>5</sup> but not of the non-inhibitory serpins<sup>6,7</sup> (notably the egg-white protein ovalbumin, whose structure<sup>8,9</sup> provides the prototype intact form shown in Fig. 1b). There is good evidence<sup>8</sup> that this failure of ovalbumin to undergo the S→R change is due to substitutions in the conserved row of alanines and small-bulk residues that form the base of the reactive centre loop of the serpins, 10–14 residues (P<sub>10</sub>–P<sub>14</sub>) from the P<sub>i</sub> reactive centre (Table 1). This is in keeping with similar findings<sup>10–14</sup> in a series of antithrombin variants identified in patients with familial thrombosis; we illustrate our findings with a non-functional antithrombin<sup>11</sup>, in which the P<sub>10</sub> alanine has been replaced by a proline (Table 1; Fig. 1e). The clue to the role of the P<sub>10</sub>–P<sub>14</sub> region came from the structure of intact ovalbumin (Fig. 1b), where it acts as a stalk that holds the reactive centre in a helical conformation inappropriate for inhibitory activity. Altogether

these observations led to the proposal<sup>9</sup> that the inhibitory activity of serpins is dependent on the ability of the loop to fold at its N-terminal stalk, and, by inference, to partially reinsert in the A-sheet to give a loop conformation near that of the canonical form (modelled in Fig. 1c).

This proposal of a reactive centre with mobility between the extreme forms of an exposed helix and a totally reinserted loop peptide, was subjected to test with native antithrombin. It was reasoned that mild denaturing conditions should destabilize the exposed helix and favour incorporation of the stalk into the A-sheet with a consequent increase in thermal stability. Guanidinium chloride was chosen as previous work<sup>15,16</sup> had shown that dilute concentrations produced a change in the fluorescence emission of serpins. The addition of 0.9M guanidinium chloride (see Fig. 1f) did indeed give a major change in the thermal stability of antithrombin comparable to, but not as great as, that which occurs on cleavage of the reactive centre. Similar results were obtained with the four other inhibitory serpins tested,  $\alpha_1$ -antitrypsin, C1-inhibitor, heparin cofactor II and  $\alpha_1$ -antichymotrypsin; but as predicted by the model, no such change in stability occurred on exposure to guanidinium chloride of ovalbumin or the P<sub>10</sub> proline variant of antithrombin.

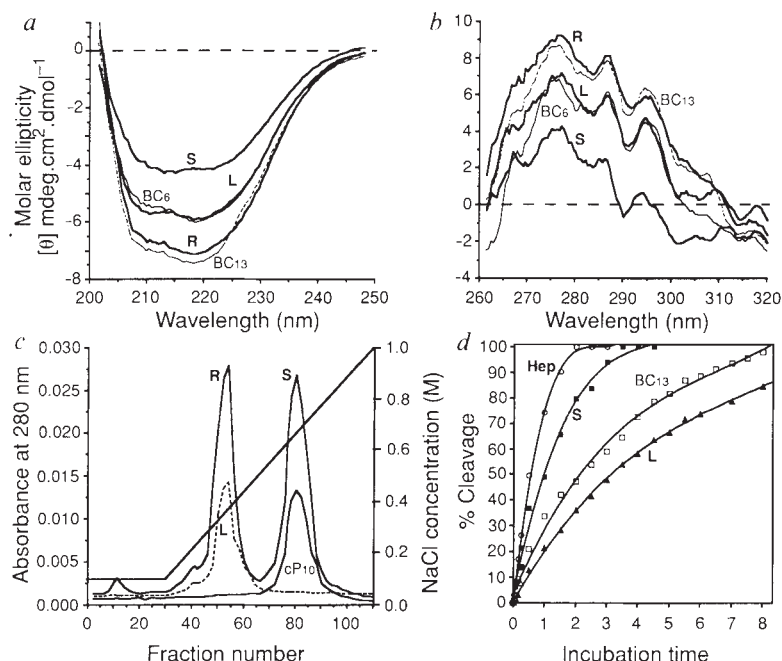
We have demonstrated with antithrombin, and confirmed with a series of other serpins, that each of the inhibitory members of the family form a new stable locked (L) conformation on exposure to dilute guanidinium chloride, that this conformation is inactive and remains unchanged even after dialysing away the guanidinium chloride and prolonged storage. Reversion to the native state, with recovery of inhibitory activity, was demonstrated with  $\alpha_1$ -antitrypsin (which has no S→S bonds) after complete unfolding and refolding from 6M guanidinium chloride. Amino-acid sequencing of L-form antithrombin and ultracentrifugation confirmed that it was intact, uncleaved and monomeric. The properties of L-state antithrombin closely resemble cleaved R-state antithrombin: it has lost its inhibitory activity, is thermostable, and has lost its high affinity-binding for heparin with transformation to a low-affinity form identical to that of cleaved antithrombin (Fig. 2c).

Supporting evidence that the intact L-state antithrombin shares a conformation similar to the R-form is provided by a peptide annealing technique<sup>17</sup>. We have applied this to produce insertions into the A-sheet of antithrombin using both the full-length peptide BC<sub>13</sub>(P<sub>2–14</sub>) and also a shorter hexapeptide, BC<sub>6</sub>(P<sub>7–14</sub>), which gave a better model of partial loop reinsertion (Fig. 1d). As Figs 1f and 2a, b show, the full peptide gave changes to the heat stability plot and circular dichroism spectra of native antithrombin that were superimposable on that of cleaved R-state antithrombin, whereas the hexapeptide gave intermediate changes superimposable with those of L-state antithrombin. Both binary complexes had lost their inhibitory activity and their high-affinity binding of heparin. These overall results support the conclusion that in the L state reinsertion of the peptide loop takes place beyond that necessary for the optimal inhibitory canonical form (Fig. 1c).

FIG. 1 A-sheet of serpins in blue, showing schematically the varying conformations of the reactive centre loop P<sub>1–14</sub> in red: a, incorporated to form middle strand S4 of sheet in cleaved R antitrypsin; b, exposed helix in intact ovalbumin; cleaved ovalbumin is virtually identical except for the loss of the helical segment of the reactive centre loop; c, model of canonical form with reincorporation of 4 residues as strand 4; as proposed, further insertion (arrowed) beyond six residues will lead to a collapse of the loop to give the inactive L form; d, annealing of synthesized loop peptides gives the binary complex forms with physical properties of the R (BC<sub>13</sub>) and L (BC<sub>6</sub>) forms, but with reactive centre inactivity and cleavage susceptibility of the helical (ovalbumin) model. e, Heat-stability curves showing melting temperatures of: S native antithrombin; R antithrombin cleaved at reactive centre; ov, superimposed native and cleaved ovalbumin; cP<sub>10</sub>, cleaved mutant P<sub>10</sub> antithrombin with proline substitution for alanine. f, Heat stability curves of S, R and L antithrombin compared with the binary complexes BC<sub>13</sub> and BC<sub>6</sub>. METHODS. Structures from coordinates of a, cleaved antitrypsin<sup>4</sup>; b, intact ovalbumin<sup>8</sup>; and d, a hybrid depiction of a and b; c, canonical model computed by C. J. Marshall using energy minimization of religated loop of antitrypsin<sup>21</sup> fixed at P<sub>6</sub> and stressed back into the sheet then allowed to relax. Heat-stability curves and reactive centre cleavage were performed as described in ref. 7. The P<sub>10</sub> proline mutant (antithrombin Cambridge)<sup>11</sup> was obtained from heterozygous plasma and purified from normal component subsequent to reactive-centre cleavage and heparin-affinity chromatography (see Fig. 2c). L-antithrombin was prepared by incubation of antithrombin in 0.9M guanidinium chloride at 4 °C for 12 h with subsequent dialysis to 50 mM sodium phosphate, 20 mM NaCl buffer, pH 7.6. The product was checked by N-terminal sequencing, gel electrophoresis, and sedimentation equilibrium ultracentrifugation. Binary complexes were formed by incubation of the synthetic peptides (Table 1) as detailed in ref. 17, with confirmation of annealing by electrophoresis and total amino-acid analysis.

FIG. 2 *a* and *b*, Circular dichroism spectra of intact S-, cleaved R-, and L-state antithrombins (bold traces) and the BC<sub>13</sub> and BC<sub>6</sub> binary complexes of antithrombin. Ovalbumin (not shown) gave no difference between intact and cleaved forms<sup>6</sup>, confirming that the circular dichroism spectra reflect changes related to the A-sheet rather than in the reactive centre helicity. *c*, Elution of mixed R and S antithrombins from a heparin-Sepharose affinity column 0.2–1.0M NaCl gradient; intact L-state antithrombin (broken line) has the same low affinity as R-cleaved antithrombin; reactive-centre cleaved P<sub>10</sub> proline mutant of antithrombin (cP<sub>10</sub>) retains the heparin affinity of intact normal antithrombin. *d*, Cleavage of reactive centre of antithrombin by subtilisin: L, L-form; BC<sub>13</sub>, binary complexed antithrombin; S, native (non-heparin) antithrombin; Hep, 1:1 molar heparinized antithrombin.

**METHODS.** Circular dichroism measurements were made as in ref. 13 using a mean residue weight of 134. Incubation of antithrombin<sup>5</sup> with subtilisin at 37 °C, pH 7.6 (20 mM NaCl, 50 mM Tris-HCl), enzyme:substrate ratio 1:2,000. Cleavage checked by N-terminal sequencing.



The identification of the new L conformation immediately explains the puzzling properties of the plasminogen activator inhibitor PAI-1. This inhibitor controls the turnover of fibrin in human plasma and variations in its concentration and activity correlate with the onset of coronary thrombosis. The puzzle has been that PAI-1 exists *in vitro* in a latent state<sup>18</sup> that can only be activated by addition to and dialysis from 6M guanidinium chloride. These properties can now be explained by its being in the L conformation, a conclusion confirmed by thermal stability data<sup>19</sup>: as expected, activated PAI-1 has characteristic serpin lability at 56 °C but latent PAI-1 has the predicted intermediate stability (80 °C) of the L conformation. The *in vivo* activity of PAI-1 depends on its binding to the plasma protein vitronectin and it follows that this binding must hold the reactive centre loop out of the A-sheet. Evidence for such linkage is provided here for the parallel binding and activation of antithrombin by heparin; Fig. 2*d* shows the increased loop exposure that occurs in the presence of heparin and Fig. 2*c* shows the changes in heparin affinity that accompany reincorporation of the loop as strand 4 of the A-sheet. Opening of the A-sheet involves not only movements of strands 2 and 3 but also of helix D (ref. 20), the primary binding site of heparin<sup>2</sup>; this displacement explains the demonstrated loss of heparin affinity in the L-form, binary complexed and cleaved antithrombins. As predicted, there is no such loss of heparin affinity in reactive-centre-cleaved P<sub>10</sub> proline antithrombin as the mutation prevents loop insertion into the A-sheet and consequently movement of the D helix. Thus in

antithrombin, as well as PAI-1, evolution has modified the linked movement of the reactive centre and the A-sheet to provide mechanisms for the modulation of inhibitory activity.

In a wider context, these observations suggest why the serpins have evolved a large and mobile reactive centre by comparison to the smaller canonical form of the other families of serine protease inhibitors. There will be an additional survival advantage in complex physiological environments in having a reactive centre that can vary its exposure to proteolytic attack. To illustrate this we examined the susceptibility of four different configurations of the reactive centre of antithrombin to cleavage by the bacterial protease subtilisin (Fig. 2*d*). As expected, the fully incorporated L state is relatively protected, as also is the helical conformation which will be induced in the binary complex. Of particular interest is the difference in the rate of cleavage of antithrombin in the presence or absence of heparin. The results are compatible with the proposal that antithrombin in the circulation has its active centre in an equilibrium between the inactive and canonical conformations—partially protected and partially active. *In vivo* this equilibrium will be tipped when the antithrombin binds to the heparans of the endothelial surface to give the fully active but vulnerable inhibitor.

**Note added in proof:** Since this manuscript was submitted, the structure of latent PAI-1 has been solved by E. Goldsmith and colleagues (University of Texas, Dallas) and shows that strand 4 is incorporated in sheet A to residue P<sub>4</sub> (E. Goldsmith, personal communication). □

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# Crystal structure of an RNA double helix incorporating a track of non-Watson–Crick base pairs

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THE crystal structure of the RNA dodecamer duplex (r-GGACUUCGGUCC)<sub>2</sub> has been determined. The dodecamers stack end-to-end in the crystal, simulating infinite A-form helices with only a break in the phosphodiester chain. These infinite helices are held together in the crystal by hydrogen bonding between ribose hydroxyl groups and a variety of donors and acceptors. The four noncomplementary nucleotides in the middle of the sequence did not form an internal loop, but rather a highly regular double-helix incorporating the non-Watson–Crick base pairs, G · U and U · C. This is the first direct observation of a U · C (or T · C) base pair in a crystal structure. The U · C pairs each form only a single base–base hydrogen bond, but are stabilized by a water molecule which bridges between the ring nitrogens and by four waters in the major groove which link the bases and phosphates. The lack of distortion introduced in the double helix by the U · C mismatch may explain its low efficiency of repair in DNA. The G · U wobble pair is also stabilized by a minor-groove water which bridges between the unpaired guanine amino and the ribose hydroxyl of the uracil. This structure emphasizes the importance of specific hydrogen bonding between not only the nucleotide bases, but also the ribose hydroxyls, phosphate oxygens and tightly bound waters in stabilization of the intramolecular and intermolecular structures of double helical RNA.

The RNA sequence GGACUUCGGUCC (including a 5'-phosphate) may form either an intramolecular hairpin loop or an intermolecular double helix containing bulges with the latter favoured by high RNA and salt concentrations. The three-dimensional structure of the hairpin loop formed by this sequence has been determined by NMR methods<sup>1,2</sup> and a structural basis for its extreme stability in comparison with other loops of the same size but different sequence has been proposed. Crystals grown in the presence of 0.2 M sodium citrate were obtained in the monoclinic space group C2. X-ray diffraction data were collected to 2.0 Å resolution and the structure determined by molecular replacement<sup>3</sup> using a canonical RNA A-form helix<sup>4</sup> corresponding to the first four base pairs as a probe. The remainder of the structure was completed by an iterative phasing and refinement process in which additional residues were added to the model and ever-improving electron density maps were calculated on the basis of the more complete model. The current *R* factor is 18% with 29 water molecules included in the model. A complete description of the structure including details of its solution and refinement will be published elsewhere (S.R.H. *et al.*, manuscript in preparation). In the crystal, the RNA dodecanucleotide forms a double helix of 12 base pairs (denoted UUCG-duplex), with one dodecamer single strand per asymmetric (unique) unit and the opposite, base-paired strand generated by a crystallographic 2-fold axis as shown schematically in Fig. 1 and in stereo in Fig. 2. Because of this symmetry only 6 base pairs are unique. Another crystallographic 2-fold axis relates two dodecamer duplexes such that they stack in a head-to-tail fashion, with the 5' and 3' ends of one molecule abutting the 3' and 5' termini of the other (Fig. 2). The result is a pseudo-infinite helix throughout the crystal with continuous base stacking and only a break in the backbone connectivity at the 5' and 3' ends of each dodecamer.

The UUCG-duplex closely approximates a standard A-form RNA helix (Fig. 3). Other examples of A-form RNA double helices, which have been observed crystallographically, are the oligoribonucleotide<sup>5</sup> [U(UA)<sub>6</sub>A]<sub>2</sub>, and the various tRNA helices<sup>6–8</sup>. The regularity of the UUCG-duplex and the similarity of its helical parameters to other known RNA duplexes is unexpected in light of its incorporation of four non-Watson–Crick base pairs.

The pseudo-infinite helices of the UUCG-duplex are linked to one another by only four direct hydrogen bonds per dodecamer. Other intermolecular interactions are through water-mediated hydrogen bonding. Significantly, all of the direct hydrogen bonds involve a ribose O2' hydroxyl either as a donor or acceptor. These observed crystal packing interactions can serve as a model for RNA–RNA interactions in complex RNA molecules such as ribosomal RNAs or ribozymes<sup>9</sup>, or for the packing of genomic RNA in the capsids of RNA viruses. The importance of the O2' hydroxyl as a glue for packing interactions between RNA helices and a potential site for disruption of RNA–RNA contacts should not be overlooked.

The most novel feature of the UUCG-duplex is the inclusion of a track of four nonstandard base pairs, G · U and U · C, in the double helix. Figure 4a shows the observed structure of the G · U pair including water molecules bound in the grooves. A bridging water between the guanine amino, N2, and the uracil ribose hydroxyl, O2', in the minor groove seems to provide more stabilization than would be possible for a G · T pair in DNA. A water was also bound to the N2 amino of the G · U pair in yeast tRNA<sup>phe</sup> and is in the appropriate position to bridge to

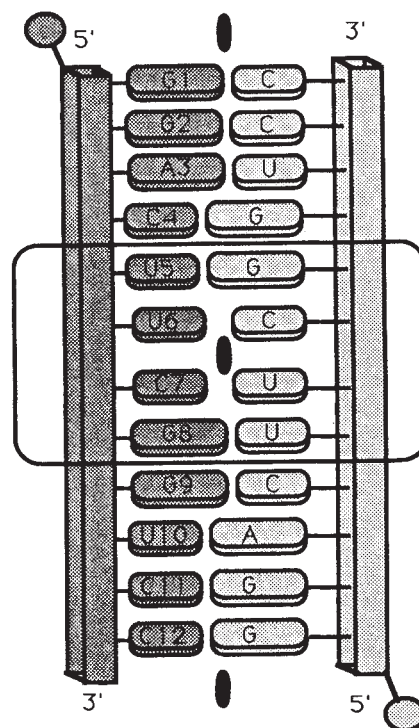


FIG. 1 Schematic diagram of the UUCG-duplex, which was identified in crystals of the dodecamer ribonucleotide (r-GGACUUCGGUCC). The phosphodiester backbone is depicted as a rectangular tube, the bases as ovals (purines, large; pyrimidines, small) and the 5'-phosphates as spheres. The darker shaded portion corresponds to the crystallographically unique portion of the structure with the lighter shaded portion generated by a crystallographic twofold axis indicated by a black oval. The four terminal base pairs on either end are formed by standard Watson–Crick pairing, whereas the middle four bases form nonstandard pairs as indicated by the bracketing. Twofold symmetry axes relating this dodecamer duplex to others stacking above and below in the crystal are also shown.

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FIG. 2 Ball-and-stick stereoview of the UUCG-duplex and another symmetry related duplex. This stacking pattern continues throughout the crystal forming a pseudo-infinite helix. The helical rotation angle for the pseudo-C-G step between dodecamers is only  $16.1^\circ$  with a rise of  $2.12 \text{ \AA}$ . These anomalous parameters may be because of the lack of chain connectivity, characteristic of a C-G step, or both.

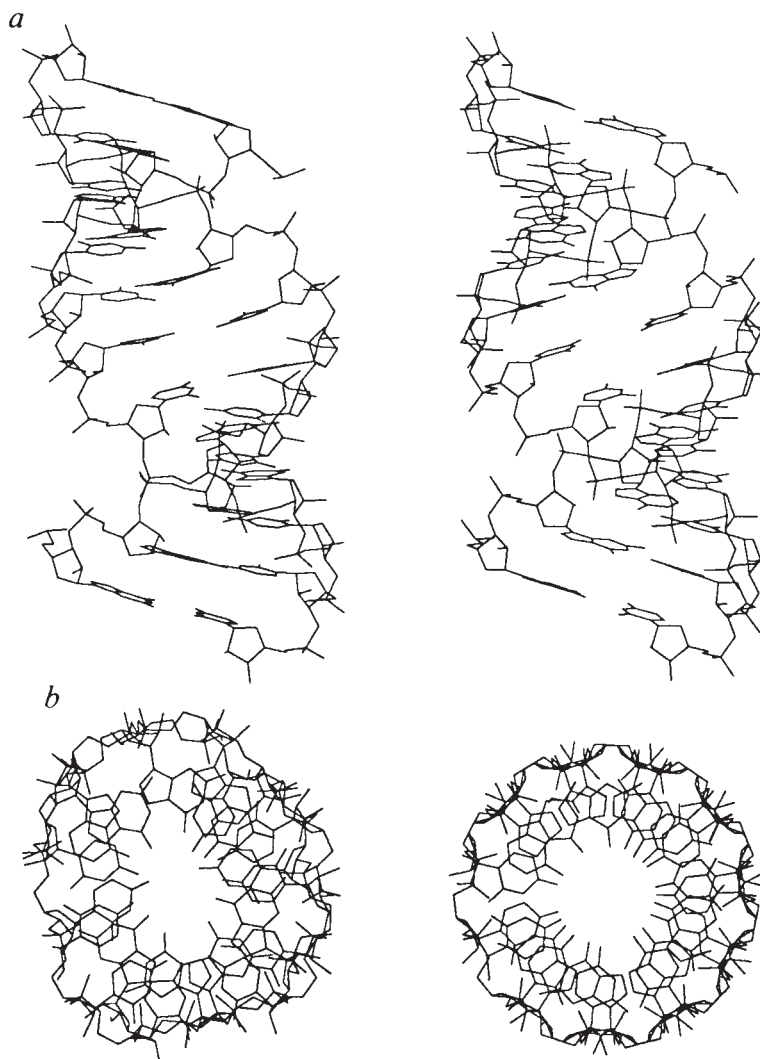
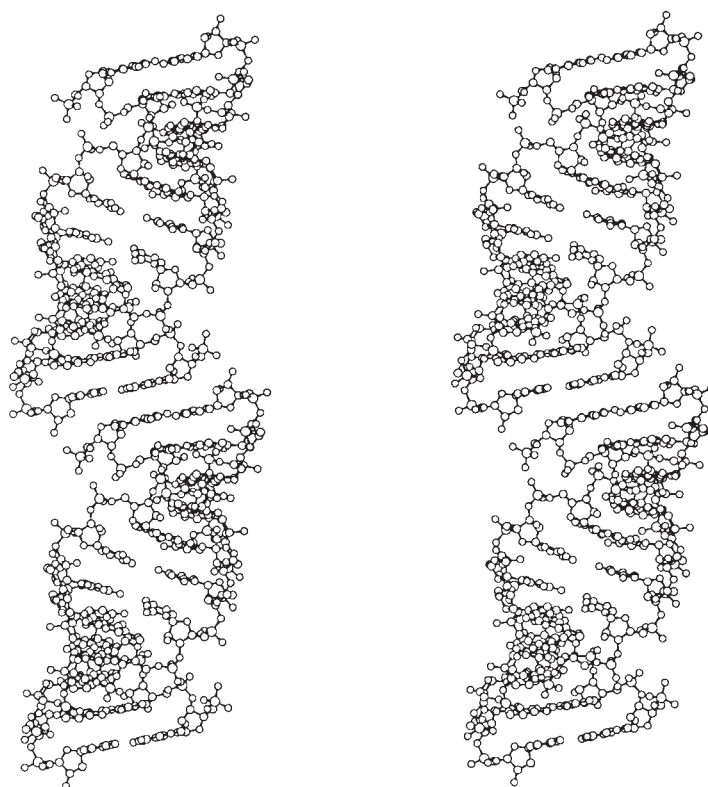


FIG. 3 Comparison of the UUCG-duplex with a standard A-form RNA helix. The average helix rotation angle of the UUCG-duplex is  $32.1^\circ$  and rise per residue  $2.93 \text{ \AA}$  compared with the values of  $32.7^\circ$  and  $2.81 \text{ \AA}$  for a canonical RNA-A helix as determined by X-ray fibre diffraction<sup>4</sup>. The average displacement of the base pairs from the helix axis is identical in the UUCG-duplex and a standard A-form RNA helix. A least-squares superposition of the UUCG-duplex with a canonical RNA-A duplex of the same sequence shows the RMS difference between these two models is  $1.48 \text{ \AA}$ . *a*, View perpendicular to the helix axis and into the major groove. The UUCG-duplex was superimposed onto the A-RNA helix and the two then translated apart. Although the overall length and width are generally the same, the UUCG-duplex (left) has a significantly wider major groove. *b*, View down the helix axes. The distortion of the UUCG-duplex cylinder is due to variation in phosphate displacement from the helix axis and varies between  $10.9 \text{ \AA}$  for the phosphate of residue C4 to  $8.1 \text{ \AA}$  for the phosphate of U6, in the U-C pair. The standard RNA-form A helix has a uniform phosphate displacement of  $8.7 \text{ \AA}$ .



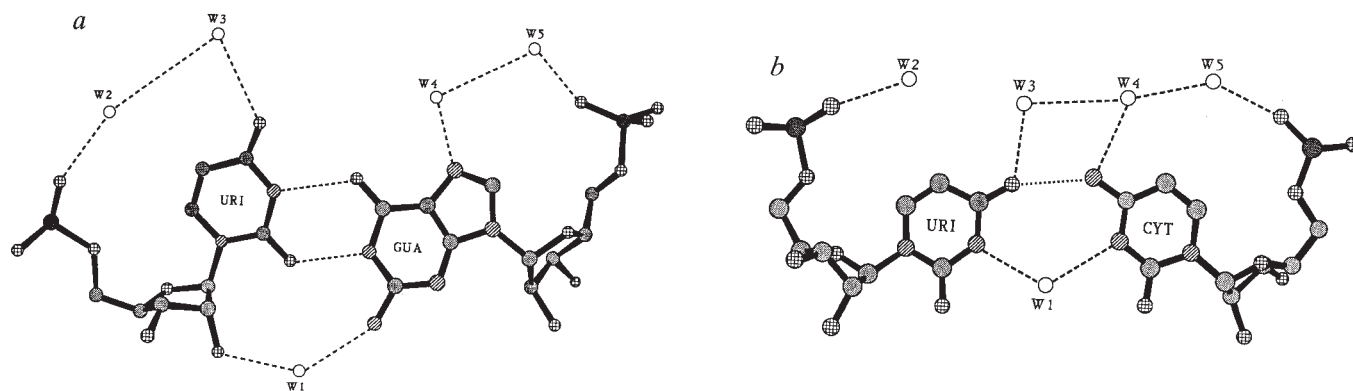


FIG. 4 *a*, The wobble G · U base pair including bound waters as observed in the crystal structure of the UUCG-duplex. Atoms are shaded according to elemental type (P, darkest solid; C, lighter solid; N, diagonal lines; O, cross-hatched lines) waters are unshaded. Base-base, water-nucleotide and water-water hydrogen bonds are indicated as dashed lines. *b*, The U · C

base pair including bound waters as observed in the crystal structure of the UUCG-duplex. The only direct base-base hydrogen bond is between U(O4) and C(N4). Element type and hydrogen bonding is indicated as in *a*. There is also hydrogen bonding between waters in the plane of the U · C pair and the twofold related C · U pair (not shown).

the O2' of the uracil on the opposite strand<sup>7</sup>. By contrast, structures of A, B and Z-form DNA incorporating G · T pairs<sup>10,11</sup> contain a bridging water in the minor groove linking the guanine N2 to the thymine O2. As the thymine O2 is also involved in wobble base-pairing, the water bridge is probably less stabilizing than that to the free ribose hydroxyl in RNA. In addition to stabilization of the G · U pair, this bound water may be important in the initial cleavage step of the self-splicing reaction occurring after the conserved U · G in group I introns<sup>12,13</sup> either by holding the uracil ribose in the necessary conformation; by acting as a proton donor, directly, or indirectly through the O2' ribose hydroxyl; or by participating along with the O2' in binding of a magnesium ion<sup>14</sup>. The importance of this bound water in the cleavage reaction may be testable by substitution of guanine with inosine which presumably would not be able to tightly bind this water in an I · U base pair.

The structure of a U · C pair (Fig. 4*b*) has not previously been observed crystallographically, however, an NMR study<sup>15</sup> of a T · C pair in a DNA duplex suggested a model which is very similar to that which we observe. To incorporate a pyrimidine-pyrimidine base pair into the helix without introducing severe radial distortion, the angle between the bases is opened so as to form only a single base-base hydrogen bond even though two are possible. This energetic loss is overcome by including a bridging water molecule between the N3 endocyclic nitrogens.

The waters involved in the minor groove bridging between the non-Watson-Crick pairs G · U and U · C are both extremely well ordered (atomic mobility parameter  $B = 17 \text{ \AA}^2$  and  $18 \text{ \AA}^2$ , respectively) and should be considered integral parts of the structure of these base pairs. The water molecules bound in the major groove of both the G · U and U · C pairs which tether the bases to the phosphate backbone are generally more mobile (average  $B = 31 \text{ \AA}^2$ ). In the G · T pairs of DNA there is a different pattern of water bonding in the major groove which does not involve the phosphate oxygens, but only the bases<sup>11</sup>.

We have considered the effects of incorporating a T · C pair of the same geometry and dimensions as observed in the UUCG-duplex into a DNA B-form duplex. Two measures of the distortion introduced by a base pair having the same geometry as the U · C pair in the UUCG-duplex are the distance between C1' atoms of cytosine and uracil (thymine) and the angle between the C1'-N1 glycosyl bonds. In comparison with canonical B-form DNA base pairs, the C1'-C1' separation is increased by  $1 \text{ \AA}$  and the angle between the glycosyl bonds is increased by  $15^\circ$ . Model building suggests that these differences can be easily incorporated in a B-form helix, although some variation in helical parameters such as helical twist may result. Gel electro-

phoretic studies of T · C pairs in a DNA duplex show little distortion of the helix on a gross level<sup>16</sup>. Chemical modification studies show T · C exposure depends on the surrounding base sequence with the 5-6 bond of thymine being available for modification when flanked by purines and inaccessible when flanked by pyrimidines<sup>16</sup>. Interestingly, this agrees with our results in the UUCG-duplex, where the C5 and C6 atoms of uracil in the U · C pairs are completely inaccessible to solvent and are flanked by pyrimidines. Crystal structures of a variety of oligonucleotides<sup>17</sup> have shown A-form DNA to have very similar helical parameters to A-RNA as opposed to earlier fibre diffraction models of A-form DNA which are more compact. Thus, an A-form DNA helix should also be able to incorporate the nonstandard base pairing in the UUCG-duplex with little distortion.

Finally, the pseudo-dyad relating the glycosyl bonds of Watson-Crick base pairs is perturbed to various degrees in mismatched pairs in DNA. It has been proposed<sup>11</sup> that mismatch repair systems recognize this asymmetry as a consequence of mispairing with an efficiency correlated with the degree of distortion. Assuming that T · C pairs in DNA have a geometry similar to the U · C pairs seen in this RNA structure, very little asymmetry is seen (as judged by the angle between the glycosidic bonds and the C6-C6 vector) agreeing with the observation that T · C pairs are generally poorly repaired in bacteria<sup>18</sup>. □

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# Mutation detection

R. G. H. Cotton and A. D. B. Malcolm

The detection and characterization of mutations in genes has become a major area of interest in many areas of biology. Such variation may account for speciation, tumour formation, drug resistance, as well as the more obvious nature of inherited disease.

As genes are cloned in increasing numbers, an increasing number of mutations in these genes is being detected. Many of these mutations are of considerable significance in human disease and, thus, the question as to how such mutations can be detected is assuming increasing clinical importance<sup>1</sup>.

This is clearly vital in the case of inherited disease but, increasingly, our understanding of the way in which such alterations in the genome can give rise to both inherited and sporadic cases of cancer, means that such techniques are assuming greater importance in oncology<sup>2,3</sup>. With pathogens such as viruses, not only is the identification of sequence variation important for epidemiological studies, but it now seems that the mechanism by which drug resistance arises may sometimes depend on sequence variation at the DNA level.

In practical terms, it is convenient to subdivide the problem into two broad areas: initial detection of unknown mutations in a gene, and later screening to detect previously described mutations in a large population. In human disease, such mutations may be polymorphisms that are merely genetically linked to a disease locus, or they may be the causative mutation itself<sup>4</sup>.

## Sequencing

All these mutations can of course be detected by direct sequencing, but in most laboratories this is not yet rapid enough to be used in a routine diagnostic context. Two recent developments promise to speed this up.

The first is the use of magnetic beads, which greatly simplifies the various purification steps (see figure). The fragment to be sequenced is end-labelled with biotin and can then be separated from the solution by the use of magnetic beads coated with streptavidin. This DNA can be used as a template for any of the various dideoxy sequencing methodologies, irrespective of the label used. As the oligonucleotide primers used in the polymerase chain reaction (PCR) can easily be labelled with biotin during synthesis, this allows direct sequencing of amplified genomic DNA<sup>5-7</sup>. The whole 'trick' depends on the strength of the biotin-streptavidin interaction which will withstand the alkaline denaturation conditions used to remove the labelled DNA

strand from the immobilized template. The hardware required is both robust and inexpensive. And, the other components can be purchased either in kit form (DynaL (UK) Ltd, Wirral, Merseyside, United Kingdom) or as individual reagents.

The second advance is the development of individual fluorescent labels for each of the four terminating dideoxynucleotides, which then enables the gel to be 'read' continuously during electrophoresis.

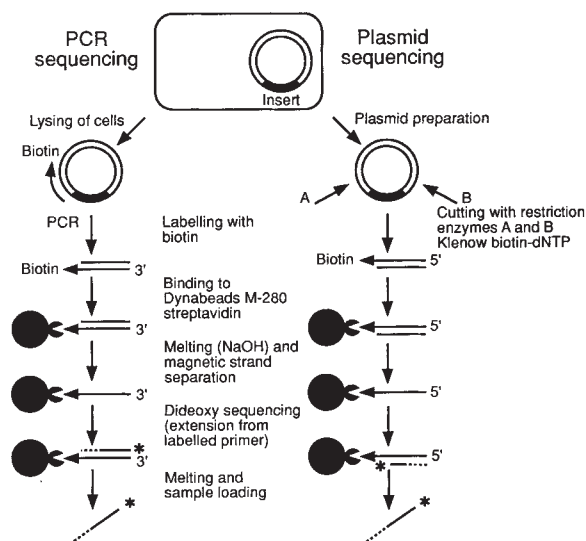
Even these two developments, however, have not obviated the need for the rapid screening of sequences simply in order to detect mutations.

## DGGE

Denaturing gradient gel electrophoresis (DGGE) involves the generation of heteroduplexes between wild-type sequence and (presumed) mutant sequence. When electrophoresed through a gel with an increasing gradient of denaturant (temperature, urea, formamide etcetera), the mismatch at the site of mutation will result in aberrant migration. The greatest advantage of this method is the comparatively small number of experimental manipulations required. This method does, however, suffer from one or two drawbacks.

First, as the ends of a duplex tend to 'fray', that is, denature earlier than would be expected on the basis of its sequence, mutations occurring close to these ends might therefore be missed. This problem can be circumvented by 'gluing' the ends together by ligating (or using terminal deoxynucleotidyl transferase) a run of Gs or Cs to produce a 'GC clamp'<sup>8</sup>.

Second, the generation of a temperature gradient in the gel requires the purchase (or construction) of special equipment, at additional expense. Denaturing gradient gel electrophoresis does, however, have one particular advantage that is not offered by other methods: the ability to identify mutations produced by mutagenesis that perhaps are present at as little as one per cent of the total sample.



## Chemical cleavage

The chemical cleavage of mismatch method is a more recent development, but is gaining rapidly in popularity<sup>9</sup>. Reference DNA probe is mixed with excess test DNA or RNA, the mixture melted, and then cooled to allow re-annealing. Heteroduplex formation takes place with a mismatch at the point of mutation. This mismatch has enhanced sensitivity towards chemical cleavage at C by hydroxylamine and T by osmium tetroxide. Subsequent cleavage at the sites of modification by piperidine and fragment sizing by electrophoresis allows the mismatch and, hence, the mutation to be identified. The slight complications that can result from the occasional artefactual cleavage suggest, however, that this primarily is a tool for scanning a large sequence. Final confirmation still requires sequencing of the (short) region thus identified. However, the particular advantages of this method include the length of screen (up to 2 kb), the ability to position multiple mutations in a particular region (between or within a stretch of nucleic acid), and the fact that it can be multiplexed to cover much larger lengths<sup>10</sup>. As with the other methods, the chemical cleavage of mismatch method can also be used in a non-radioactive mode<sup>11</sup>. When it was first developed, some mutations were difficult to detect and could be missed. Recent improvements to the protocol, however, now allow 100 per cent detection with confidence<sup>12</sup>. The most 'unpleasant' disadvantage of this method is the



need, on safety grounds, to use a fume hood when handling osmium tetroxide and piperidine. Would-be users may wish to be reminded that the name 'osmium' derives from the Greek for 'the smelly one'!

### Carbodiimide

A variation of this involves the use of carbodiimide modification of mismatched G and T bases. The latest version of this relies on blocking, by the chemically-modified bases, of primer extension<sup>13</sup>. The greatest advantage of this method is that it uses only a single chemical. The most obvious disadvantage is that the TG mismatch containing the target bases is the most stable of all the various mismatched base pairs. To date, however, this method has not been widely used and therefore a full description of the pros and cons is not yet available.

### Screening

Early success with the rapid detection of known mutations was achieved by Y. W. Kan and colleagues with the detection of the sickle mutation by a *Hpa* I restriction enzyme polymorphism<sup>14</sup>. Subsequently, the use of allele-specific oligonucleotides was developed principally by R. B. Wallace and colleagues<sup>15</sup>. Progress in both these areas has been facilitated greatly by the recent introduction of PCR.

Wallace has now developed a protocol that reduces problems of cross reactivity that are associated with allele-specific oligonucleotide hybridization. This is achieved using a 30-fold excess of a 'cold' competitor. Excess unlabelled oligonucleotide then prevents the labelled oligonucleotide from hybridizing to the alternative allele<sup>16</sup>.

Tetra-alkylammonium ions can be used to reduce the dependence of the melting temperature on base composition. This means that identical hybridization conditions can be used for several different oligonucleotides. It is this that has allowed the development of 'reverse' dot blots, whereby many different oligonucleotides are immobilized to the membrane. Amplified, labelled genomic material is then allowed to hybridize to it. In this way, 26 different HLA alleles can be studied in one experiment, and the most common mutations giving rise to cystic fibrosis can be tackled in a similar manner.

Two elegant methods rely on the oligonucleotide hybridization being permissive or restrictive to PCR amplification. The first of these, amplification refractory mutation system or 'ARMS' places the mismatch in the penultimate position of the primer<sup>17</sup>. Under appropriate conditions, only the fully matched oligonucleotide leads to a product, which then needs to be viewed on a gel.

## Biotechnica briefs

About 450 exhibitors from 20 countries are expected to attend Biotechnica '91, the International Trade Fair for Biotechnology that is to be held 22–24 October in Hannover, Germany. A new gel capillary for capillary electrophoresis and an inverted confocal microscope are among the exhibits.

ACCORDING to Eppendorf, automated cell injection, electrode calibration, tip cleaning and cell location memory are just a few of the uses for the company's new Micromanipulator 5171 (*Reader Service No. 101*). The micromanipulator, which was developed by Eppendorf in collaboration with researchers at the Max-Planck Institute, Germany, features a programmable joystick/keypad and is designed to provide a resolution of 0.15  $\mu\text{m}$ . Drift-free stepper motors allow stable measurements for hours or days, Eppendorf says. In addition, the joystick may be used to control independently a second device, including a second Manipulator 5171 or a motorized microscope stage.

Using the Uvikon 900 series of double-beam UV/visible spectrophotometers made by Kontron Instruments, users can simultaneously measure the rate of up to ten enzymatic reactions using a thermostatted automatic cell changer (*Reader Service No. 102*). An electrically-controlled heating system in the spectrophotometer maintains the temperature of the samples to within 0.1  $^{\circ}\text{C}$ , Kontron says. The progression of the reaction is displayed in real time on the built-in VDU in either mono or colour. The kinetics software can then be used to calculate the rate of reaction, either in

real time or by the recall of post-run data stored on a floppy or hard disk. Recalculation of any time period is possible with the data saved in ASCII format.

### Ideas in immunology

To simplify cytokine testing in all murine systems, Endogen has introduced four new Murine Cytokine ELISA Kits: Murine IL-4, Murine IL-6, Murine GM-CSF and Murine TNF (*Reader Service No. 103*). According to Endogen, these ELISAs can be used to measure levels of natural and recombinant murine IL-4, IL-6, GM-CSF or TNF in serum and culture supernatants. The ELISAs are dual-antibody immunometric sandwich ELISAs. Plate reagent is added to the wells along with diluted standards and samples. During a two-hour incubation, cytokine is captured and bound to the solid phase. Conjugated antibody is added and, following a further one-hour incubation, the cytokine is quantitated by an enzyme reaction. The resulting colour change is detected using a standard ELISA reader. With the Murine IL-4 and GM-CSF ELISAs, Endogen says it is possible to detect less than five picograms of cytokine per millilitre, while the IL-6 and TNF ELISAs can detect 15 picograms and less than 25 picograms of cytokine per millilitre, respectively. Using the kits, users can run

A particularly attractive development of this, labels the two oligonucleotides with different colours. A non colour-blind scientist can then identify the two homozygotes and the heterozygote by the colour in the Eppendorf tube at the end of the amplification. Thus, only a few more steps are required before 'do-it-yourself' genotyping kits become available over the counter. □

R. G. H. Cotton is at the Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Flemington Road, Parkville, 3052, Melbourne, Australia and A. D. B. Malcolm is in the Department of Biochemistry, Charing Cross and Westminster Medical School, Fulham Palace Road, London W6 8RF, United Kingdom. For more information, fill in reader service number 100.

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up to 44 samples in duplicate at one time, or two partial plates on separate occasions.

The Affinica Antibody Orientation Kit from Schleicher and Schuell uses high-capacity Affinica Protein-A agarose in combination with an optimized buffer system to **isolate and immobilize antibodies via their Fc region** (*Reader Service No. 104*). According to S & S, no



S & S's antibody orientation kit.

prepurification of the antibody sample is required. Then, using a dimethyl suberimidate crosslinker, which is provided with the kit, the antibody is bound covalently to the Protein A, under mild conditions and in an orientation that maximizes the antigenic binding capacity of the gel, S & S says. No oxidation of your antibody population is necessary. The immunoaffinity column that results is designed to provide high antigenic yields. The kit contains all you need to produce five 1-ml immunoaffinity columns.

## Oligos to order

Perform synthetic oligonucleotide purity runs in 5–40 minutes, says Beckman, with the company's eCAP U100P **gel capillary** for the P/ACE or other capillary electrophoresis (CE) systems (*Reader Service No. 105*). Using this capillary, the CE technique can be used to provide automated, high-resolution analyses of primers and probes, and antisense therapeutic agents. This CE set-up provides



Perform oligo purity checks with capillary electrophoresis — the slab-gel substitute.

online detection and, as Beckman points out, eliminates the labour-intensive tasks that are associated with slab-gel electrophoresis, namely staining, radiolabelling, scanning and autoradiography. The new eCAP U100P capillary contains urea, a denaturant that has been shown to prevent the formation of secondary and tertiary structures, to minimize peak

compression and optimize resolution. The capillaries can be reused and can be trimmed to optimize speed and resolution. For example, Beckman says a 20-cm capillary can be run for speed, a 30-cm capillary for higher resolution, or a 40-cm capillary for very high resolution. The eCAP U100P gel capillary is available in kit form, which includes everything you need to get started — gel capillaries, test mix, markers, buffers and vials. Gel capillaries are also available individually for use with Beckman's P/ACE System, as well as other commercial and home-built systems.

Oligoclean from United States Biochemical is designed to provide an inexpensive alternative to HPLC as a method of **purifying oligonucleotides** (*Reader Service No. 106*). USB says Oligoclean can be used to remove protecting groups and small failed products from  $\leq 85$  O.D. units of synthetic oligonucleotides in as little as 45 minutes. This, USB says, is faster than with PAGE purification, although both methods offer comparable yields. With Oligoclean, sample preparation is not necessary, that is, oligos can be purified from the cold ammonium hydroxide hydrolysate. Oligos recovered in this way are ready for use without additional work-up. Oligoclean is sold in 100-ml quantities for \$85 (US).

## At the molecular level

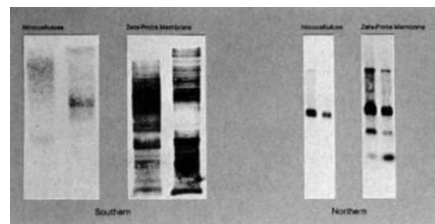
Applied Biosystems has introduced 40-nmol polystyrene columns as a replacement for controlled pore glass **synthesis supports on DNA synthesizers** (*Reader Service No. 107*). The new polystyrene support material, a hydrophobic organic polymer, is designed to offer mechanical stability, rapid reaction kinetics and the ability to be washed quickly and efficiently with organic solvents. In addition, phosphoramidites can now be used at a double dilution of 0.05 M. This, ABI says, can translate into savings of about 25 per cent on the cycle cost. Use of polystyrene columns also extends the life of diluted phosphoramidites on the instrument from two to three weeks. The 40-nmol columns can be used on all of ABI's DNA synthesizers.

Instacryl, the non-toxic **protein electrophoresis matrix** from International Biotechnologies, is designed to provide linear separation of proteins from 14 kD up to and greater than 300 kD (*Reader Service No. 108*). The matrix is a water-soluble copolymer that forms a gel in the presence of oxygen and without exposure to toxic chemicals. In addition, Instacryl gels can be run in either a horizontal or vertical format. Proteins resolved using Instacryl gels can be stained using coomassie blue, silver stain, or a non-fixative copper stain. The recovery of discrete

proteins can be accomplished by solubilizing the gel with periodic acid, IBI says. As Instacryl is supplied as a stock solution that hardens with dithiothreitol, contact with acrylamide, bisacrylamide, ammonium persulphate and TEMED is eliminated.

## More on membranes

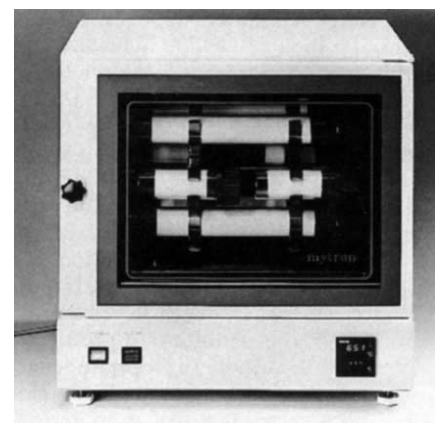
Bio-Rad has introduced new Zeta-Probe **charge-modified membranes**, which the company recommends for use in all types of nucleic acid blotting of DNA and RNA for subsequent hybridization, and also for some protein blotting applications (*Reader Service No. 109*). The membranes have a capacity that is 5- to 10-fold greater than nitrocellulose, Bio-Rad says, and will bind both native and denatured DNA. Low ionic-strength



Strong signals from Bio-Rad's Zeta-Probe charge-modified membranes.

binding and transfer buffers can be used because, unlike nitrocellulose, Zeta-Probe membranes bind nucleic acids independent of salt concentration, Bio-Rad says. This characteristic permits electrophoretic transfer of nucleic acids to Zeta-Probe membranes. The membrane is capable of binding fragments as small as six bases. Fragments as small as 20 bases can be detected by hybridization. Nucleic acids are bound tightly, allowing at least 10 rounds of hybridization on a single membrane, the company says.

Dispense with the plastic bag method of performing hybridizations, Biometra has introduced a Mini Hybridization Oven that is designed for the simple and reproducible **hybridization of northern, Southern, dot, slot or colony blots** in bottles (*Reader Service No. 110*). A



Biometra's solution to hybridization.



special design feature of the hybridization oven is the option of tilting the rotor axis. This provides a tumbling action to the bottles and ensures optimal wetting and incubation of the membranes, Biometra says. This set-up is also designed to minimize the amount of hybridization solution required. A double-glass vacuum door provides optimal heat insulation and a rotor stop activates automatically when the door is opened. Although the oven has a small footprint, it can hold six large bottles (300 mm × 35 mm) or 12 small bottles (150 mm × 35 mm or 100 mm × 35 mm). Each Mini Hybridization Oven is supplied with four large bottles and meshes.

### Applications software

ImageQuant v.3.15 is the latest software upgrade of an **image analysis software package** from Molecular Dynamics that is designed for use with the company's PhosphorImager computing densitometer and personal densitometer (*Reader Service No. 111*). The package includes the new application module, DNA Sequencing, which allows researchers to call primary sequence data directly from sequencing gel images by pointing and clicking on the bands using a mouse. According to Molecular Dynamics, this eliminates the need to write down the sequence as it is being read, and the need to enter the sequence into a computer file. Both of these refinements are designed to alleviate the tedium and cut down on the number of errors introduced. Additional features include a new set of manual baseline software tools that are designed to give users complete control over electrophoretogram peak integration, a reorganized and expanded online help system that offers point-and-click access to help screens and automatically suggests related help topics, and a set of new operating manuals.

Biosym's **NMR structure determination tools** are a set of programmes for structure generation, analysis and refinement that are based on multidimensional NMR data (*Reader Service No. 112*). This new suite of programmes should provide the NMR spectroscopist with a comprehensive set of tools for generating and refining molecular structures based on the primary sequence and NMR restraints derived from multidimensional NMR experiments, Biosym says. The first five programmes, which will be available in December on both Silicon Graphics and IBM RISC 6000 workstations, include: NMR Database, for coordination of spectroscopic and molecular data; DG II and Simulated Annealing for the generation of structures; IRMA for the refinement of structures; and NOE Analysis for the evaluation of structures. All of these programmes are accessed

through Insight II, the company's workstation-based graphical interface.

### Giving it a whirl

With the addition of a 1-kD and 50-kD version, Filtron now supplies eight molecular weight cut-offs in its **Microsep microconcentrator** line (*Reader Service No. 113*). The line-up now includes the following molecular weight cut offs: 1 kD, 3 kD, 10 kD, 30 kD, 50 kD, 100 kD, 300 kD and 1,000 kD. The 1-kD device can be used to concentrate or desalt low molecular weight biomolecules such as peptides, oligonucleotides and growth factors. For concentrating and desalting



Microconcentrators: in 1- and 50-kD cut-offs.

monoclonal antibodies, purifying nucleic acids and separating primers from amplified DNA, Filtron recommends its 50-kD device. Each device is supplied preassembled and is disposable, and each incorporates an Omega low protein adsorption membrane that is sealed in the device without the use of an O-ring. This set-up minimizes adsorption and extends the range of chemical compatibility, Filtron says. A concentrate collector ring in the bottom of the sample reservoir is designed to prevent the sample from concentrating to dryness. Sample recovery can be achieved by reverse spinning the concentrate into the concentrate cup of the device, or by extracting the concentrate with a conventional pipette.

The MS 60 and MS 80 are two new **ultracentrifuges** in the MSE Series from



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Sanyo Gallenkamp, which offer maximum speeds of 60,000 and 80,000 r.p.m., respectively, and a maximum RCF of 605,000 g (*Reader Service No. 114*). Both units are powered by frequency-controlled, induction drive systems that are accurate to  $\pm 20$  r.p.m., even at full speed, Sanyo says. The refrigeration system uses R-22 refrigerant in a closed system. Temperatures can be controlled over the range of 0–45 °C,  $\pm 0.1$  °C using a radiometer detection device, Sanyo says. Thirty two user programmes and the facility to maintain automatically a rotor log book are two additional design features. The MS Series features a host of safety features including over-speed protection, imbalance detection over the entire speed range, and over-temperature detection.

### Picture perfect

Leica's CLSM-Fluovert is an inverted version of the company's upright confocal laser scanning microscope (CLSM) (*Reader Service No. 115*). The **inverted confocal microscope** has been designed with maximum fluorescence yield in mind. The number of optical elements

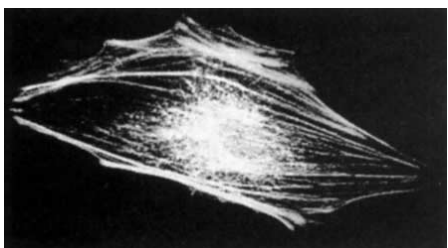


Image formation using Leica's new inverted confocal microscope.

has been minimized: the microscope incorporates only two highly reflective dielectric mirrors and two optical elements in the emission pathway. This results in a very light-sensitive system with an optical transfer efficiency of 0.89, Leica says. To improve ergonomics and accessibility, Leica has integrated the confocal part of the microscope into the anti-vibration table and provided space on either side of the instrument for experimental set-ups. Other features include two focusing

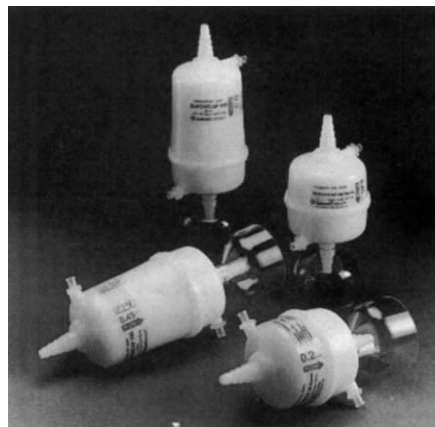
modes (Galvanometer drive z stage with a 40-nm step size and motorization of the fine focus drive of the Fluovert), optimized simultaneous dual-wavelength excitation, a special time linescan function for capturing very fast events, and an extensive software library for two- and three-dimensional processing. The microscope can be used with a range of lasers: Ar-ion, Kr/Ar and HeNe.

The Seescan Sprite **mains stabilizer**, which can be used with any microscope power unit up to 200 W, is designed to improve the consistency and accuracy of research results in the area of microscope photography and image analysis (*Reader Service No. 116*). In tests carried out at Seescan, the company determined that although image analysis can be used to make measurements with high precision, the quality of the video and computing technology is not often matched by that of the illumination source used to light the sample. The Sprite stabilizer can accept input from normal laboratory mains (both 200–250 V/50 Hz and 80–160 V/60Hz versions are available) and outputs a stabilized voltage. An interference suppression unit has been fitted to ensure that other laboratory equipment is not affected. The stabilizer unit costs £800 (UK).

### Tools of the trade

SLT Labinstruments has developed a new **screening plate for cell cultures** that is designed to provide optimum viewing conditions under the microscope (*Reader Service No. 117*). The optical features of the rectangular well bottom with its lens form provide a visual field covering the entire width of the well bottom. Disturbing reflections are eliminated, SLT says. In addition, the wells are coated to assist cell growth and to prevent the formation of air bubbles. The plate can be used in the automated reading of cell cultures using the General Cell Screening System, an automated cell culture analysis package that is based on photometric cell counting without the need for the pretreatment of cells.

According to Gelman Sciences, high throughput and high retention efficiencies are the hallmark of the company's new SuporCap and CritiCap **capsule filters** (*Reader Service No. 118*). Both capsules contain Supor, an inherently hydrophilic polysulphone microporous membrane that is designed to offer low protein binding and low extractables. In



Capsule filters from Gelman.

addition, both units incorporate the 'Acrolak' vent design to ensure convenient and safe venting, as well as a removable filling bell that protects the filtrate from contamination during filling applications, Gelman says. The SuporCap 50 and CritiCap 50 have effective filtration areas of 500 cm<sup>2</sup> and are recommended for volumes of up to 50 litres. The SuporCap 100 and CritiCap 100, however, have filtration areas of 1,000 cm<sup>2</sup> and are designed to handle volumes of up to 100 litres. SuporCap capsules are available in 0.2- and 0.45- $\mu$ m pore sizes, with an upstream layer of 0.8  $\mu$ m membrane for maximizing throughput with difficult-to-filter solutions. The CritiCap capsules incorporate a double layer of 0.2- $\mu$ m membrane to maximize particle retention. □

*These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.*

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